WORLD HEALTH ORGANIZATION
INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

Cell Differentiation, Genes and Cancer

Edited by

T. Kakunaga, T. Sugimura, L. Tomatis and H. Yamasaki

IARC Scientific Publications No. 92

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International Agency for Research on Cancer
Lyon
1988

Published by the International Agency for Research on Cancer,
150 cours Albert Thomas, 69372 Lyon Cedex 08, France

Distributed by Oxford University Press, Walton Street, Oxford OX2 6DP, UK
Distributed in the USA by Oxford University Press, New York

ISBN 92 832 1192 8
ISSN 0330–5085

Printed in the United Kingdom

CELL DIFFERENTIATION, GENES AND CANCER

INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

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.

The publications of the Agency are intended to contribute to the dissemination of authoritative information on different aspects of cancer research. A complete list is printed at the back of the book.

This volume comprises the proceedings of a symposium held in Osaka on 24–26 February 1987, under the sponsorship of Osaka University and the International Agency for Research on Cancer.

Cover

Photomicrograph prepared by microinjection of fluorescent dyes into cultured cells. In the presence of the tumour-promoting agent 12-*O*-tetradecanoylphorbol 13-acetate (TPA), the intercellular transfer of dye molecules is inhibited, so the pattern of cells injected remains clear; in the absence of TPA, the dyes would rapidly spread across all the culture. The cells have been microinjected in a pattern that provides a representation of a growth factor or TPA binding to a cellular receptor and transfer of second messages to the nucleus. (Prepared by F. Katoh, IARC, see article on p. 57).

Foreword

The relatively early hypothesis of the role of aberrant differentiation in the development of tumours has, to a certain extent, been revisited and revised by recent research in molecular biology. A variety of differentiation markers have been found which are aberrantly expressed in human experimental tumours. Abnormal differentiation pathways could be the basis for increased susceptibility to carcinogens or may develop at an early stage in the process of carcinogenesis. A change in the genes controlling the intrinsic differentiation process is probably an essential early event in transformation, while other differentiation-related events may play a major role in tumour promotion.

The IARC and Osaka University jointly organized an international symposium to review and discuss recent progress in the field of cell differentiation and carcinogenesis, a field in which basic research plays the necessary essential role. Among the participants of the symposium were some of the world's leading experts and the present proceedings represent, therefore, a timely updating of this fast-moving area of research. The IARC is particularly indebted to the local organizers, and especially to Professor Yamamura and Professor Kakunaga of Osaka University for their invaluable help and expert advice, and the marvellous facilities they provided. We would also like to thank Professor T. Sugimura for his support, interest and active participation in the Symposium.

L. Tomatis, M.D.
Director, IARC

Takeo Kakunaga (1937–1988)

As this volume went to press, we were distressed to hear of the untimely death of Dr Takeo Kakunaga on 21 September 1988, from lung cancer. He was one of the organizers of the IARC–Osaka Symposium on Cell Differentiation and Cancer from which the present proceedings resulted and had many friends at the IARC, with which he extensively collaborated. He was also serving as a member of the Fellowships Selection Committee of the IARC. We regret that he died before seeing the publication of this book, the fruit of our most recent collaboration.

Dr Kakunaga's sudden death is a great loss to the cancer research community. The originality of his approach to research and the warm sincerity of his personality will be missed by all who knew him. We send our deep condolences to Mrs Mariko Kakunaga and share the sorrow of Dr Kakunaga's death with her.

Lorenzo Tomatis

Hiroshi Yamasaki

Introduction

Recent rapid advances in techniques and concepts of molecular biology coupled with improvements in knowledge of cell biology have transformed certain aspects of cancer research. In particular, several genes which are considered critically involved in carcinogenesis have been identified; some of them are called oncogenes. From the view that carcinogenesis is a process of disturbed cell differentiation, it was not so much a surprise as a pleasing confirmation for cancer researchers to know that normal functions of cellular oncogenes are to control cell differentiation and proliferation. The international symposium co-organized by the International Agency for Research on Cancer and Osaka University was convened to discuss the recent advances in understanding the involvement of cell differentiation and critical genes in carcinogenesis.

In this book, critical gene expression, including oncogene expression, is discussed as a determinant of cell behaviour and of the different stages of carcinogenesis. Identification and characterization of tumour suppressor genes has also helped to clarify the relationship between cell differentiation and carcinogenesis. Since cells receive various signals from the environment through their membranes, studies of cellular membranes at the molecular level are also an important area for research. The pathway of signals from the membrane to the nucleus, the signal transduction pathway, is another field of intense research interest.

It was obviously not possible to cover all details of these fast-moving topics, but we have assembled important papers which survey all of the major aspects of current research into the links between cell differentiation, genes and carcinogenesis.

Contents

Oncogenes and cellular genes regulating differentiation and carcinogenesis

ABERRANT DIFFERENTIATION IN CARCINOGENESIS

Aberrant differentiation in mouse skin carcinogenesis

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Introduction

Cancer is commonly considered a disease in which rapid, unregulated proliferation causes the unbridled expansion of tumour cells to a clinically relevant endpoint. Some oncogenes have been recognized as related to growth factors or growth-factor receptors. However, the transformation-sensitive target cells for these transduced 'proliferation' genes have generally been simple cell types (fibroblasts) or cells which are already abnormal by virtue of a previous treatment or extended cultivation *in vitro*. *In vivo*, the usual target sites for cancer development in humans or carcinogen-exposed experimental animals are epithelial cells. Commonly, lining epithelia such as those of the skin, bronchus, gastrointestinal tract, pancreatic duct, mammary duct or urogenital tract are involved. These epithelia are complex in structure, and many have a stringently regulated programme of differentiation which can counteract aberrant proliferative activity. For example, psoriasis is a hyperproliferative disease in skin which is not neoplastic because the intact programme of epidermal differentiation inevitably leads to the death of the hyperproliferative cells. Such considerations suggest that the carcinogenesis process requires an alteration in the programme of terminal differentiation in addition to aberrant growth control. The development of a differentiation abnormality must constitute a rate-limiting step in cancer development in lining epithelia.

A number of biological observations support this idea. Hyperproliferation alone could not account for the long latency period in carcinogenesis. Epithelial cancers are commonly preceded by metaplastic and dysplastic lesions whose major phenotype is an alteration in differentiation (Decosse, 1983). Certain malignant tumours have relatively low rates of proliferation (e.g., chronic lymphocytic leukaemia), while benign tumours may have high rates of proliferation (e.g., skin papilloma). Almost all malignant tumours express alterations in the expression of normal markers of differentiation. Multipotential malignant stem cells, such as those in teratocarcinomas, have the capacity to revert to the non-neoplastic state by the induction of a programme of terminal differentiation (Pierce and Wallace, 1971).

Aberrant differentiation of a fundamental type may occur early in carcinogenesis. Patients carrying the autosomal dominant gene for multiple polyposis have a genetic predisposition to develop intestinal tract cancer and are considered to be constitutively 'initiated' (Kopelovitch *et al.*, 1979). Examination of non-neoplastic colonic mucosa from these patients indicates that the normal pattern of epithelial differentiation is altered and epithelial cells continue to proliferate in crypt regions restricted to non-proliferative maturing cells in the normal colon (Deschner & Lipkin, 1975). This is also the earliest documented lesion during the induction of colon cancer in mice and rats by chemical carcinogens (Deschner, 1978).

Fundamental alterations in the regulation of differentiation are early events in other models for experimental carcinogenesis. Skin papillomas differ from normal skin by the presence of proliferating cells in the strata normally confined to differentiating phenotypes (Yuspa, 1985a). The loss of proliferative potential normally coupled to phenotypic changes in the differentiating cell strata is not obligatory in benign neoplastic epidermal cells (Yuspa *et al.*, 1981). Similarly, the earliest change noted during chemical induction of rat mammary tumours is an inhibition of the normal differentiation of terminal end buds into alveolar buds. The consequent intraductal proliferation of cells blocked in maturation potential results in a microtumour (Russo *et al.*, 1983). Delay of carcinogen exposure until the completion of terminal end bud differentiation in mature or multiparous rats markedly reduces the tumour yield (Russo *et al.*, 1983).

Cell-culture experiments have confirmed that the uncoupling of differentiation and proliferation controls is central to cancer development and occurs at an early stage. The malignant myeloid leukaemia cell phenotype requires that cells both establish themselves as independent from exogenous growth regulators and insensitive to differentiation signals (Sachs, 1980). Mouse 3T3 preadipocytes are converted to malignancy only after distinct regulatory sites for differentiation control are altered (Scott & Maercklein, 1985). Mouse keratinocytes are resistant to the differentiation signal of elevated extracellular Ca^{2+} if they have been exposed to carcinogens *in vitro* (Kulesz-Martin *et al.*, 1980) or isolated from mouse skin initiated by chemical carcinogens *in vivo* (Yuspa & Morgan, 1981) or from benign skin tumours (Yuspa *et al.*, 1986).

Support for a requisite role of aberrant differentiation in cancer development has come from studies indicating that the malignant phenotype can be reversed or suppressed by inducing differentiation. Injection of mouse embryonal carcinoma cells into blastocysts will give rise to normal chimaeric mice (Brinster, 1974). Cell hybrids of highly malignant HeLa cells and normal human fibroblasts or keratinocytes are often non-tumorigenic when injected *in vivo*. Analysis of the injection site reveals that hybrid cells are induced to terminally differentiate in the host animal and assume the phenotype of their normal parent, either fibroblasts or keratinocytes (Stanbridge *et al.*, 1982). This restoration of an intact differentiation programme by experimental methods can reverse the malignant phenotype. During the induction of liver cancer by chemical carcinogens, preneoplastic hepatic nodules are known to disappear spontaneously. This remodelling of preneoplastic hepatocytes occurs as a result of redifferentiation of altered cells to mature hepatocytes (Tatematsu *et al.*,

1983). Regressing skin papillomas may undergo an analogous spontaneous change (Burns *et al.*, 1976). It can be assumed that restoration of an intact differentiation programme in the early stages of carcinogenesis may be an endogenous mechanism to protect against cancer development. However, little is known of the factors which regulate differentiation in lining epithelia or how they may be altered in neoplastic cells.

Experimental studies

The induction of tumours on mouse skin has been one of the major models from which modern concepts of chemical carcinogenesis have been derived. This model has revealed the multi-step nature of the tumour-induction process, the irreversibility of early carcinogen-induced changes, the sequential requirements for exposure to initiating and promoting agents, the stepwise progression from preneoplastic to neoplastic phenotypes, potential reversibility of preneoplastic lesions, and the ability of sequential carcinogen exposure to enhance malignant progression (Yuspa, 1986). More recently the use of epidermal cell culture systems in association with biochemical and molecular biological techniques has contributed to a cellular and molecular understanding of some of the changes which underlie the biology of tumour development (Yuspa, 1985b). Since exposure of normal mouse skin or normal keratinocytes to chemical carcinogens causes a defect in the programme of epidermal differentiation which allows cells to escape some signals for terminal differentiation (Yuspa & Morgan, 1981), this may be a particularly useful model to examine the underlying changes in differentiation associated with carcinogenesis.

In 1980, it was first reported that extracellular Ca^{2+} regulates the differentiation of normal keratinocytes *in vitro* (Hennings *et al.*, 1980). Subsequently, a number of other studies from a variety of laboratories have confirmed and extended the initial observations to suggest that Ca^{2+} acts as a signal to trigger other changes which are permissive for differentiation to proceed (Yuspa, 1985b). Additional changes include modifications of the cell membrane and receptors (Strickland *et al.*, 1984),

Table 1. Differentiation programme of keratinocytes

	Keratinocytes *in vivo*		Keratinocytes *in vitro*		v-Ras^H keratinocytes *in vitro*	
	Basal	Suprabasal	Low Ca^{2+}	High Ca^{2+}	Low Ca^{2+}	High Ca^{2+}
Proliferation	+	−	+	−	+	−
K14	+	+	+	+	+	+
K1	−	+	−	+	−	−
K10	−	+	−	+	−	−
Filaggrin	−	+	−	+	−	+
CE	−	+	−	+	−	+
Transglutaminase	−	+	−	+	−	+
Pemphigus	−	+	−	+	−	−
Pemphigoid	+	−	+	−	+	+
Cornified cells	−	+	−	+	−	±

intracellular ion distribution (Hennings *et al.*, 1983), and the expression of differentiation-specific genes (Roop *et al.*, 1988). Additional studies suggested that phorbol ester tumour promoters could also induce epidermal differentiation in the absence of a modification of extracellular Ca^{2+} (Yuspa *et al.*, 1982), suggesting that Ca^{2+} and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) may have some common action to signal epidermal cells to differentiate.

When epidermal cells are grown under Ca^{2+} conditions below 0.1 mM, they are phenotypically similar to basal epidermal cells (Table 1). Elevation of extracellular Ca^{2+} above 0.1 mM leads to vertical stratification and a rapid change in morphology and biochemistry which is irreversible after 48–72 hours. Within 3–7 days stratifying cells form mature squames which ultimately slough from the culture dish (Table 1). When epidermal basal cells are exposed to initiating carcinogens, cellular foci evolve which are resistant to the Ca^{2+} signal and although stratified, cells in these foci continue to proliferate under high Ca^{2+} concentrations (Kulesz-Martin *et al.*, 1980). Since similar cellular foci can be derived from mouse skin initiated *in vivo* and subsequently selected in culture by an increased extracellular Ca^{2+} (Yuspa & Morgan, 1981), it is presumed that these cells represent initiated cells. Furthermore, cells isolated from chemically induced squamous papillomas (Yuspa *et al.*, 1986) exhibit a similar cell-culture phenotype; the cells continue to proliferate in the higher Ca^{2+} medium. Such cells are fully capable of stratification and terminal differentiation, since some cells in these cultures form squames and slough. However, the substrate-attached population continues to proliferate (Yuspa *et al.*, 1986). This is similar to the differentiation pattern of papillomas *in vivo* (Yuspa, 1985a) and carcinogen-induced foci *in vitro*. Malignant epidermal cells also show a pattern of Ca^{2+} resistance (Yuspa *et al.*, 1980). Importantly, a pattern of Ca^{2+} resistance has also been reported for tumour-derived cells of human skin and bronchus (Brysk *et al.*, 1984; Lechner *et al.*, 1983).

A number of studies *in vivo* and *in vitro* have suggested that activation of the *ras*^{H} gene by point mutation is sufficient to initiate carcinogenesis in mouse epidermis (Bizub *et al.*, 1986; Brown *et al.*, 1986; Quintanilla *et al.*, 1986; Roop *et al.*, 1986). When an activated *ras* oncogene is introduced into normal keratinocytes, recipient cells are resistant to Ca^{2+}-induced differentiation. Under high Ca^{2+} culture conditions, cells with an activated *ras* oncogene remain in a late basal cell stage of differentiation (Table 1) (Yuspa *et al.*, 1985). The cultured cells express basal cell markers, but have a reduced capacity to proliferate. This finding strongly links a defect in the response to Ca^{2+} as a differentiation signal with the initiation of carcinogenesis. Recent evidence has demonstrated that a Ca^{2+} gradient exists across the epidermis *in vivo*, with basal cell Ca^{2+} levels being low and granular cell Ca^{2+} levels high (Malmquist *et al.*, 1984; Menon *et al.*, 1985). Thus, Ca^{2+} may be a regulator of epidermal differentiation *in vivo*. If so, then a defect in response to Ca^{2+} might be very relevant to the selection of initiated cells and formation of tumours *in vivo*.

The strength of the evidence surrounding an association of aberrant differentiation and tumour formation in skin carcinogenesis has generated a substantial research effort, both to characterize the mechanism by which Ca^{2+} and TPA act to

induce differentiation and to develop markers which are useful to characterize various differentiation states in the epidermis (Roop *et al.*, 1985).

One obvious link between the two effectors comes from studies indicating that an increase in intracellular Ca^{2+} catalyses phosphatidylinositol (PI) turnover yielding an increase in inositol phosphates and diacylglycerol. It would be anticipated that a rise in diacylglycerol would stimulate the translocation and activation of protein kinase C. Such a pathway could explain the parallels between Ca^{2+}-induced differentiation and the induction of differentiation by phorbol esters (Yuspa *et al.*, 1983). Recently, it has been shown that the pattern of changes in epidermal protein synthesis and protein phosphorylation which occur within one or two hours after exposure to either increased extracellular Ca^{2+} or the tumour promoter TPA are similar, although a wider spectrum of changes is caused by the tumour promoter (Wirth *et al.*, 1987). Furthermore, it has been shown that exogenous phospholipase C and exogenous diacylglycerol can mimic the action of the phorbol ester tumour promoters on epidermal differentiation (Jeng *et al.*, 1985). All of these results suggest a pathway regulating differentiation which involves exposure to increasing concentrations of extracellular Ca^{2+}, increasing intracellular Ca^{2+} as a consequence, the activation of an endogenous phospholipase C and the stimulation of phosphatidylinositol metabolism leading to the activation of protein kinase C.

It could be postulated that initiation of carcinogenesis in mouse epidermis is associated with a change in Ca^{2+} transport or in the Ca^{2+} requirements of enzymes involved in PI metabolism. Our studies suggest that PI metabolism is stimulated in both normal and initiated keratinocytes (Jaken & Yuspa, 1988). Since Ca^{2+} ionophores, such as A23187 or ionomycin, can stimulate PI metabolism to the same extent as an increase in extracellular Ca^{2+}, a rise in intracellular Ca^{+} is likely to be essential to activate this pathway. Additional studies indicate that ionomycin induces differentiation in the absence of an increased extracellular Ca^{2+} concentration and this is enhanced by combined treatment with TPA. Together these results indicate that a rise of intracellular concentrations of Ca^{2+} and a stimulus which activates protein kinase C could account for many aspects of the triggering for epidermal differentiation. In corollary, a defect involving one or both of these signalling processes could account for aberrant differentiation in neoplastic cells.

Extracellular Ca^{2+} concentration and the control of specific markers of differentiation

Recent findings emphasize the importance and specificity of Ca^{2+} control over keratinocyte differentiation. Measurements of epidermal Ca^{2+} *in vivo* (Malmquist *et al.*, 1984; Menon *et al.*, 1985) suggest that a gradual increase in extracellular Ca^{2+} would reflect the Ca^{2+} gradient *in vivo*. When individual markers of epidermal differentiation were studied *in vitro*, it became clear that expression was dependent on specific Ca^{2+} concentrations in the culture medium. As markers of epidermal differentiation, specific antibodies were generated against unique peptide sequences of specific keratin proteins (Roop *et al.*, 1984). Additional antibodies were made to the major protein component of the cornified envelope (here called anti-CE) and to