REVIEW Jpn. J. Cancer Res. **84**, 1091–1100, November 1993

Why Are Human Cells Resistant to Malignant Cell Transformation *in vitro*?[1]

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Transformation of human cells, both induced and spontaneous, is an extremely rare event, whereas rodent cells are relatively easily transformed when treated with a single carcinogenic agent. The present review addresses the question of why human cells are resistant to malignant transformation *in vitro*. To facilitate understanding of the problem, the process of transformation is divided operationally into two phases, i.e. phase I, immortalization; and phase II, malignant transformation. In human cells, one-phase transformation, i.e., the consecutive occurrence of phases I and II due to the action of a single carcinogenic agent, is observed only rarely. Once human cells are immortalized, however, malignant transformation by chemical carcinogens or oncogenes proceeds, suggesting that for human cells, phase I immortalization is a prerequisite for such transformation to take place. To date, about 20 papers have been published describing protocols for the two-phase transformation of a variety of human epithelial cells and fibroblasts. In most experiments, SV40, human papilloma viruses and their transforming genes are utilized for induction of phase I (immortalization) followed by the use of chemical carcinogens or activated oncogenes for induction of phase II (malignant transformation). Possible mechanisms that would render human cells refractory to transformation are discussed below.

Key words: Human cell — Transformation — Immortalization — Carcinogen — Oncogene

Since human studies *in vivo*, other than epidemiological and clinical ones, are ethically unacceptable, the use of cultured human cells represents the most promising experimental approach. In particular, a knowledge of malignant cell transformation *in vitro* should facilitate our understanding of carcinogenesis in human beings.

Malignant cell transformation of rodent cells *in vitro* was achieved with polyoma virus first (1959–1960), followed by transformation with chemical carcinogens in the mid-1960s. Reports of human cell transformation came much later, however, appearing only in the late 1970s. Successful *in vitro* transformation of the human cell by a single carcinogenic agent is an extraordinarily rare event and it was often impossible for other laboratories to reproduce findings even when using the same or similar protocols. It is thought that many data concerning unsuccessful, or non-reproducible transformation studies have been generated but remain unpublished, and it is believed therefore that human cells are rarely transformed *in vitro*, although the reasons for this are not known. The present review addresses directly the question of why human cells are resistant to malignant cell transformation *in vitro*.

In this article, the transformation process is divided operationally into two phases, i.e., phase I, immortalization; and phase II, malignant transformation. When malignant transformation is achieved without specific induction of immortalization by an agent, we refer to it as "one-phase transformation" (Fig. 1). If immortalization is a prerequisite of malignant transformation, the whole process is referred to as "two-phase transformation" (Fig. 2).

Transformation of human cells has been reviewed previously by DiPaolo,[2] Chang,[3] Rhim[4] and Shay *et al.*[5] The proceedings of a symposium on "Neoplastic transformation in human cell culture" have also been published.[6]

One-phase Transformation of Rodent Cells: a Commonly Observed Event

The first successful transformation *in vitro* of rodent cells was achieved during 1959 and 1960 by infecting SHE[3] cells with polyoma virus. Transformation of these and other rodent cells by chemical carcinogens was reported independently by the laboratories of Sachs,[7,8] Kuroki,[9,10] Kakunaga[11] and Heidelberger[12] in the mid-1960s.

[1] This review was presented at the Second International Symposium on "Theories of Carcinogenesis" which was held on 15–21 August 1992 in Oslo, Norway. A summary version of this article was included in the Proceedings.[1]

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[3] The abbreviations used are: SHE cells, Syrian hamster embryo cells; NQO, 4-nitroquinoline-1-oxide; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; HPV, human papilloma virus; EBV, Epstein-Barr virus; 5MC, 5-methylcytosine.

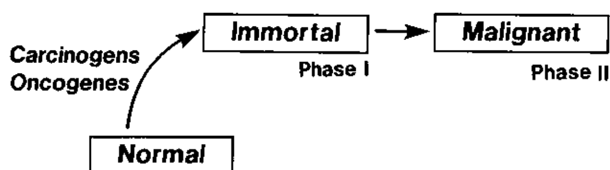

Fig. 1. One-phase transformation. Rodent cells are relatively easily transformed into malignant cells by treatment with chemical carcinogens or oncogenes, but this is a rare event with human cells.

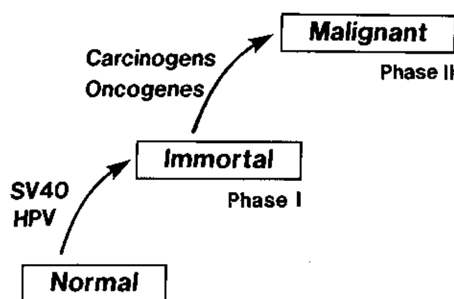

Fig. 2. Two-phase transformation. In human cells, immortalization by SV40 or HPV is a prerequisite for malignant cell transformation by carcinogens or oncogenes.

Treatment of normal SHE cells in primary or secondary culture with chemical carcinogens resulted in conversion to the malignant state within 2–3 months. Typically, transformation of SHE cells could be achieved by exposure to 4-hydroxyaminoquinoline-1-oxide for 15 minutes.[10] Although untreated SHE cells ceased dividing after day 30, the carcinogen-treated cells continued to grow, being immortalized after day 40 and eventually becoming tumorigenic after 3 months in culture. In this and other experiments with SHE cells, immortalization occurred prior to malignant transformation and without the requirement for a specific agent. This type of transformation is referred to as "one-phase (phase I plus II) transformation" (Fig. 1).

Subsequently one-phase transformation by various carcinogens was achieved in several types of rodent cells including a variety of epithelial cells. This is a commonly observed event.

One-phase Transformation of Human Cells: a Rare Event

The successful transformation of rodent cells prompted attempts to transform human cells by use of the protocol for one-phase transformation. After many attempts and few successes (summarized in Table I) it was concluded that, unlike rodent cells, one-phase transformation of human cells is a rare event.

Table I. One-phase Transformation of Human Cells

Cell type	Agent	Reference
Fibroblasts	Radiation	Borek[67]
Bronchial epithelial cells	v-Ha-ras	Yoakum *et al.*[16]
Embryo kidney cells	Adenovirus 12	Whittaker *et al.*[68]

The first purportedly successful *in vitro* transformation of human cells was reported by Kakunaga in 1978.[13] Human skin fibroblasts were treated with NQO or MNNG and transformed phenotypes such as anchorage-independent growth and morphological alteration were observed on days 60–88 after treatment. Tumorigenicity in nude mice was observed on days 107–150. Ten years after Kakunaga's report, however, these transformed cells were found to have been derived not from the normal human fibroblasts to which carcinogens had been applied, but from the human fibrosarcoma cell line 8387, established in 1966.[14, 15]

One-phase transformation of human cells by oncogenes is also a rare event (Table I). In most cases, dominant oncogenes or oncogene-carrying viruses, do not cause immortalization or malignant transformation of human cells. One of the few cases observed concerns immortalization and malignant transformation of human bronchial epithelial cells by the Harvey *ras* oncogene.[16] Transfected cells overcame the crisis which occurred in untreated cells at 3 months and continued to grow, showing transformed phenotypes and eventually forming tumors in nude mice. A marked change in karyotype was also noted. In this experiment, the *ras* gene seemed to trigger a cascade of events leading to malignant transformation, probably due to the induction of genetic instability.[17]

Failure of one-phase transformation does not imply that human cells are not competent to be transformed by chemicals, viruses or oncogenes. We have previously shown that human epidermal keratinocytes in primary culture are capable of activating chemical carcinogens, repairing DNA damage and binding phorbol ester tumor promoters.[18] These observations imply that certain capabilities of chemical transformation are inherent in human keratinocytes.

All these observations show that one-phase transformation is not applicable to human cells, and it is concluded that in order to obtain successful transformation the established protocol requires modification.

Immortalization of Human Cells

Three decades ago, Hayflick and Moorhead[19] demonstrated that human diploid fibroblasts are unable to be cultured *in vitro* for more than 10 months or 60 popula-

tion doublings. Spontaneous immortalization following senescence is an extremely rare event in human fibroblasts and epithelial cells (Table II), although it occurs commonly in rodent cells with varying frequencies depending on the species from which the cells are derived. In order to immortalize human diploid cells, for example, Namba *et al.*[20, 21] had to expose them to more than 10 treatments with either NQO or gamma-irradiation. With the exception of three reports,[16, 22, 23] activated *ras* genes have not been found to be able to immortalize human fibroblasts or epithelial cells.[21, 24–28]

From the mid-1980s, DNA tumor viruses such as SV40 and HPV have been used for immortalizing human cells, especially human epithelial cells (Table III). The large T-antigen protein encoded by the early region of the SV40 genome is responsible for immortalization of human epithelial cells. Immortalized cells by SV40 or an SV40-adenovirus 12 hybrid include epidermal keratinocytes,[26] uroepithelial cells,[29] mammary epithelial cells[30] and liver parenchymal cells.[31]

Human diploid fibroblasts, however, are rarely immortalized by SV40. Infection of human fibroblasts with SV40 results in extension of their life span *in vitro* for about 20–30 population doublings but eventually they become senescent. Wright *et al.*[32] defined the normal and extended life spans as M1 (mortality stage 1) and M2 (stage 2), respectively. SV40 allows the cells to escape from the M1 phase, but progression from the M2 stage to immortalization is a rare event, being estimated as 3×10^{-7}.[33]

From amongst more than 60 HPVs, HPV16 and HPV18 are known to immortalize human epithelial cells such as epidermal keratinocytes[34–37] and cervical epithelial cells,[38, 39] immortalization being due to the E6 and E7 regions of the HPV genome. However, human fibroblasts are not immortalized by HPV, showing an extended life span only.[35]

Human B lymphocytes are efficiently immortalized by EBV: 10–100% of the EBV-infected B cells yield progeny that can proliferate indefinitely. Among about 100 genes encoded in EBV, nuclear and membrane antigen genes,

Table II. Spontaneous Immortalization of Human Cells

Cell type	Reference
Fibroblasts	Mukherji *et al.*[69]
Epidermal keratinocytes	Boukamp *et al.*[70]
Mammary epithelial cells	Soule *et al.*[71]

Table III. Immortalization of Human Cells

Cell type	Agent	Reference
Fibroblasts	NQO	Namba *et al.*[20, 21]
	Radiation	Namba *et al.*[20, 21]
	SV40	Radna *et al.*[72]
		Shay and Wright[33]
		Wright *et al.*[32]
	myc	Morgan *et al.*[73]
Epidermal keratinocytes	HPV16	Dürst *et al.*[34]
		Pirisi *et al.*[35]
	HPV16 E6+E7	Münger *et al.*[36]
	HPV18 E6+E7	Hudson *et al.*[37]
	Ad12-SV40	Rhim *et al.*[26]
Bronchial epithelial cells	Ad12-SV40	Reddel *et al.*[74]
Tracheal epithelial cells	SV40	Gruenert *et al.*[75]
Cervical epithelial cells	HPV16, 18	Woodworth *et al.*[38]
		Pecoraro *et al.*[39]
Prostate epithelial cells	SV40	Kaighn *et al.*[76]
Uroepithelial cells	SV40	Christian *et al.*[29]
Mammary epithelial cells	SV40	Chang *et al.*[30]
	HPV16	Band *et al.*[77]
	BP	Stampfer and Bartley[78]
Kidney epithelial cells	Nickel	Tveito *et al.*[79]
Liver parenchymal cells	SV40	Namba *et al.*[31]
Ciliary epithelial cells	SV40	Coca-Prados and Wax[80]
Endothelial cells	v-*ras*, v-*mos*	Faller *et al.*[22]
Kidney tubule cells	v-*ras*	Nanus *et al.*[23]

BP: benzo[*a*]pyrene.

termed EBNA-1, -2 and LMP, are required for the virus to immortalize human B cells.[40]

Thus, although SV40, HPV and EBV DNA tumor viruses can immortalize certain human cells, the efficiency with which this is done differs markedly according to cell type. However, so far no mechanism to explain the cell-type specificity in response has been proposed. Assigning functions to these viral genes appears to be the best way of understanding the molecular and cellular events needed for immortalization.

Two-phase Transformation of Human Cells

Immortalized human cells in general exhibit certain phenotypic changes including altered morphology, a higher saturation density, growth in low-serum medium and anchorage-independent growth. They are not, however, tumorigenic when injected into nude mice. Immortalization, therefore, is accompanied by partial transformation, but further changes are needed to obtain fully malignant phenotypes.

When chemical carcinogens or activated oncogenes are applied to immortalized cells, malignant transformation is achieved as summarized in Tables IV and V. Rhim *et al.*[26, 41] for example, immortalized and transformed malignantly human epidermal keratinocytes by infecting them with a hybrid of adenovirus 12 and SV40 followed by infection with Kirsten-murine sarcoma virus or treatment with a chemical carcinogen (NQO or MNNG). Malignant transformation of human uroepithelial cells was achieved only when SV40-immortalized cells were treated with 4-aminobiphenyl, a human bladder carcinogen, 3-methylcholanthrene or EJ *ras* oncogene.[29, 42, 43] In these experiments, either chemical carcinogens or EJ *ras* alone could not immortalize human cells, suggesting that immortalization by SV40 is a prerequisite for malignant transformation. Two-phase transformation of human cells is thus a commonly observed event (Fig. 2).

It appears from the above data that immortalization is a key step in malignant transformation in human cells. Although this is also the case in rodent cells, the importance of immortalization has been ignored, because rodent cells are malignantly converted without a distinct immortalization phase and without the aid of recognized inducers of immortalization.

Table IV. Two-phase Transformation of Human Cells by Carcinogens and Radiation

Cell type	Phase I	Phase II	Reference
Fibroblasts	v-myc	BPDE	Yang *et al.*[81]
Epidermal keratinocytes	Ad12-SV40	NQO, MNNG	Rhim *et al.*[41]
		Radiation	Thraves *et al.*[82]
Uroepithelial cells	SV40	MCA	Reznikoff *et al.*[42]
	SV40	ABP	Bookland *et al.*[43]

BPDE: benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide, MCA: 3-methylcholanthrene, ABP: 4-aminobiphenyl.

Table V. Two-phase Transformation of Human Cells by Oncogenes

Cell type	Phase I	Phase II	Reference
Fibroblasts	SV40	K-ras	O'Brien *et al.*[83]
	v-myc	T24ras	Hurlin *et al.*[84]
	Radiation	H-ras	Namba *et al.*[24]
Epidermal keratinocytes	Ad12-SV40	Ki-MSV	Rhim *et al.*[26]
		v-fes, v-fms	Rhim[4]
		v-src, v-erbB	
		EBV-LMP	Fahraeus *et al.*[85]
Bronchial epithelial cells	SV40	v-Ha-ras	Amstad *et al.*[86]
Cervical epithelial cells	HPV-16	v-H-ras	DiPaolo *et al.*[87]
Uroepithelial cells	SV40	EJras	Christian *et al.*[88]
Mammary epithelial cells	Spont.	c-Ha-ras	Basolo *et al.*[89]
	BP	v-ras, v-mos	Clark *et al.*[90]
		SV40LT	
Liver parenchymal cells	SV40	Ha-MSV	Namba *et al.*[31]

EBV-LMP: EB virus latent membrane protein.

Molecular Mechanisms of Immortalization

An understanding of the molecular mechanisms underlying immortalization should provide essential information on human cell transformation *in vitro.* Two broad hypotheses can be proposed to explain the phenomenon of cellular senescence: (1) random accumulation of damage and (2) genetically programmed processes. Pereira-Smith and Smith[44,45] addressed this question and proved by hybridizing normal human fibroblasts with immortalized cell lines that the latter mechanism operates. The hybrid cells that resulted had a limited life span and could be classified into four immortal complementation groups, indicating that the immortal state is recessive. Immortalization might result from recessive changes in growth inhibitory genes involved in cellular senescence.

Of the suggested mechanisms for inhibition of cell growth, two lines of evidence support the implication of tumor suppressor genes in cellular senescence. Firstly, the p53 and Rb tumor suppressor genes have been found to bind to DNA tumor virus oncoproteins, such as the large T antigen of SV40 and E6 and E7 of HPV, all of which mediate their immortalizing functions.[46-49] Such binding may result in a perturbation of the normal regulatory functions of tumor suppressor genes.

Further evidence is available from studies with fibroblasts from hereditary cancer patients, e.g. those with Li-Fraumeni syndrome, which is associated with a mutated p53 gene transmitted through the germ line,[50] and familial polyposis coli, associated with mutations in the MCC and APC tumor suppressor genes. Bischoff *et al.*[51] reported that fibroblasts from seven of eight Li-Fraumeni syndrome patients escaped senescence and were immortalized spontaneously. These cells were assumed to be heterozygous (+/−) for p53 gene mutations. Chen *et al.*[52] found that fibroblasts from familial polyposis coli showed delayed senescence *in vitro* when cultured with a feeder layer of 3T3 cells. All these results implicate the action of tumor suppressor genes in prevention of cellular immortalization. Indeed, Hara *et al.*[53] reported that targeting these genes with anti-sense oligomers resulted in an extended life span of human fibroblasts.

Further possible mechanisms to explain cellular senescence include a decrease in degree of DNA methylation and length of telomere sequence. 5MC in DNA appears to be an important modulator of gene expression. There are several lines of evidence indicating that gradual loss of 5MC is relevant to cell senescence *in vitro* (for review, see Ref. 54). Wilson and Jones[55] demonstrated that 5MC levels decrease during cellular senescence of fibroblasts, while cells immortalized by SV40 are stably methylated over several hundred cell divisions.

Recently, attrition of telomere sequence has been proposed as a possible mechanism of senescence. Telomere sequence is an essential structural element of the ends of chromosomes and protects them from degradation or fusion. Telomeres of human cells seem to act as a mitotic clock, shortening with cell senescence *in vitro* as well as aging *in vivo.*[56-59]

Human Cells versus Rodent Cells

Spontaneous transformation is an extremely rare event in human cells, whereas rodent cells are relatively easily immortalized in the absence of any causative agent. However, there is no good explanation for this difference between human and rodent cells. As summarized in Table VI, human beings have 30-fold longer maximum life span than mice *in vivo*, and the same is true for their cells in culture. Levels of 5MC decline during cellular senescence but the rate of decrease is much slower in human cells than in mouse cells.[55] Human cells form fewer oxidative lesions than rodent cells owing to their lower oxygen consumption and more efficient repair system.[60-62] The mouse genome contains 8–16 times more telomere sequence than the human genome and, unlike human cells, its size in mouse cells seems to remain largely unchanged during passage *in vitro.*[63] In mouse cells, however, the number of complementation groups for cellular senescence is not known.

The mechanisms mentioned above, with the exception of those involving the telomere, may explain, but only in part, the difference observed between human and mouse

Table VI. Comparison of Human Cells with Mouse Cells in Cellular Senescence and Its Possible Mechanisms

	Human cells in comparison with mouse cells	(human vs. mouse)
Life span	Longer maximum life span *in vivo*	(100 years vs. 3 years)
	More population doublings *in vitro*	(60 vs. 10)
DNA methylation	Longer turnover of 5MC	(0.5–1.0% vs. 3.5% fall per cell cycle)
Telomere	Shorter telomere, decreasing with age	(10 vs. 150 kilobases)
DNA damage	Less oxidative damage	(0.4 vs. 5.5[a] thymine glycol/kg/day)

a) A value obtained with rats.

cells in their ability to undergo spontaneous transformation or immortalization.

In vivo versus in vitro

Cancer is prevalent in human beings. From one-fourth to one-third of the population die of cancer in developed countries where the life span is close to the expected maximum. Nevertheless, why is induction of cancer a rare event in cell culture? The answer may be that there are many more cells in the human body as compared to the number in a Petri dish. The total number of cells in a body is likely to be in the order of 10^{13}, while that in a cell culture experiment rarely exceeds 10^7. Possibly, the answer may lie in that the life span of a human being as compared to the duration of cell culture results in a longer time of exposure to carcinogenic agents and a longer time for expression of transformed cells: 60 years or more *in vivo* versus one year or less *in vitro*.

One of the most likely answers to the question posed above is that stem cells are present *in vivo*, but not *in vitro*. These cells are defined as cells with the capacity for extensive self-maintenance throughout the entire life-span of the organism.[64] Stem cells divide asymmetrically: one daughter cell provides for a differentiating lineage and one remains as a stem cell at a clearly defined site in the tissue architecture. *In vivo*, there are three main types of cell population in tissue: stem cells, transit cells that divide symmetrically and static cells that are being differentiated and have no proliferative potential (Fig. 3). However, cultured populations contain proliferating transit cells only. In the case of epidermal keratinocytes, for example, stem cells may be carried over from the tissue during initiation of the primary culture, but seem to be eliminated afterwards, possibly due to the absence of a specific site for their establishment and/or of growth factors allowing their maintenance *in vitro*. Certain stem cells seem to escape cellular senescence *in vivo* and *in vitro* under appropriate conditions. For example, embryonal stem cells (ES cells) are able to divide permanently when cultured in the presence of leukemia inhibitory factor (LIF).[65, 66] They are, in principle, regarded as immortalized.

The absence of stem cells *in vitro* does not account for the resistance of human cells to malignant transformation, since the situation is the same in rodent cells. However, elucidation of the molecular and cellular mechanisms of stem cells would afford new insights into carcinogenesis *in vivo* as well as *in vitro*.

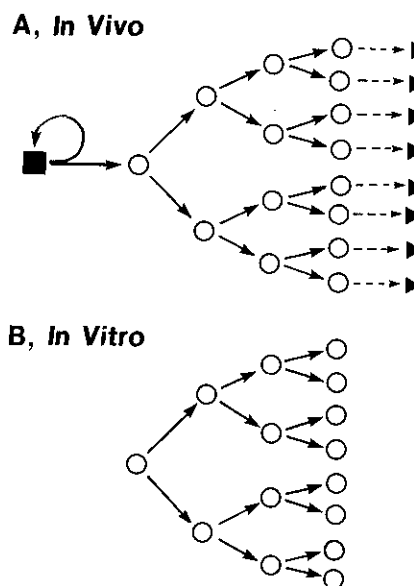

Fig. 3. Presence and absence of stem cells *in vivo* (A) and *in vitro* (B), respectively. There are three populations in tissues *in vivo*; stem cells (closed square), transit cells with the potential to divide (open circles), and static cells that are being differentiated (closed triangles). The cell population in culture, however, consists of growing transit cells and lacks stem cells.

ACKNOWLEDGMENTS

We appreciate the valuable comments of the members of the Ad Hoc Committee on "Human Cell Transformation," supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan. We also thank Dr. F. Hunter for editing the manuscript.

(Received May 31, 1993/Accepted August 12, 1993)

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