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Karyotypic analysis in the process of immortalization of human cells treated with 4-nitroquinoline 1-oxide

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Abstract

The establishment of a model system of neoplastic transformation of normal human cells has been attempted with a chemical carcinogen, 4-nitroquinoline 1-oxide (4NQO). In the course of these experiments, it was noticed that immortalization of human cells is a multi-step process involving several mutational genetic events. Thus, chromosomal changes which occurred during the process of immortalization of human fibroblasts were examined. To accomplish immortalization, fibroblasts obtained from an embryo were repeatedly treated with 10_{-6}M4NQO from primary culture to passage 51 (59 treatments in total). Before immortalization, some chromosomes (especially, chromosomes 2, 6, 8, 10, 11, 12, 15, 19, and 20), were lost at a relatively high frequency. After immortalization, the chromosomes distributed so broadly in the triploid to hypotetraploid region without a distinct modal number or without marker chromosomes that it was difficult to identify the specific chromosomes related to the immortalization of human cells. No specific structural chromosomal changes were detected. Although the significance of such chromosome changes in relation to immortalization is not clear, the loss of some specific chromosomes suggests that genes which are involved in cellular aging and which suppress immortalization may have been lost in the immortalization process.

KEYWORDS: human cells, chromosomes, aging, immortalization, 4NQO

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Karyotypic Analysis in the Process of Immortalization of Human Cells Treated with 4-Nitroquinoline 1-Oxide

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The establishment of a model system of neoplastic transformation of normal human cells has been attempted with a chemical carcinogen, 4-nitroquinoline 1-oxide (4NQO). In the course of these experiments, it was noticed that immortalization of human cells is a multi-step process involving several mutational genetic events. Thus, chromosomal changes which occurred during the process of immortalization of human fibroblasts were examined. To accomplish immortalization, fibroblasts obtained from an embryo were repeatedly treated with 10^{-6} M 4NQO from primary culture to passage 51 (59 treatments in total). Before immortalization, some chromosomes (especially, chromosomes 2, 6, 8, 10, 11, 12, 15, 19, and 20), were lost at a relatively high frequency. After immortalization, the chromosomes distributed so broadly in the triploid to hypotetraploid region without a distinct modal number or without marker chromosomes that it was difficult to identify the specific chromosomes related to the immortalization of human cells. No specific structural chromosomal changes were detected. Although the significance of such chromosome changes in relation to immortalization is not clear, the loss of some specific chromosomes suggests that genes which are involved in cellular aging and which suppress immortalization may have been lost in the immortalization process.

Key words: human cells, chromosomes, aging, immortalization, 4NQO

Many lines of evidence indicate that carcinogenesis is a multistep process. Development of a reproducible *in vitro* human cell transformation system should be particularly valuable in elucidating the mechanism in the multi-step carcinogenesis of human cells. Over the past two decades, considerable effort has been made to transform human cells (1-3). However, no reproducible human transformation system has been reported and neoplastic transformation of human cells remains a rare event. The failure of human cells to undergo transformation as readily as rodent cell lines may reflect a significant difference in the frequency of immortalization (4). Rodent cells are relatively easily immortalized in culture without any treatment with chemical carcinogens but human cells are not.

We have attempted to establish a model of neoplastic transformation of human cells with chemical carcinogens and with irradiation (1, 2, 5-7). In these trials, we noticed that once human cells are immortalized they are relatively easy to transform into neoplastic cells (2, 5). Our results are consistent with those reported by Rhim *et al.* (8, 9) and strongly indicate that immortalization is a critical step in the neoplastic transformation of human cells.

Immortalization and cellular aging are considered to be genetically regulated (10-12). Therefore, we attempted to assess which chromosomal changes are implicated in the immortalization process of human cells treated with 4NQO.

Materials and Methods

Cells and cultures. A normal human fibroblast strain, OUMS-24, was established in our laboratory from a 6-week-old female embryo. Details of the culture methods have been described elsewhere (7).

Treatment of cells with 4NQO. Details of the treatment have been described by Bai *et al.* (7). Briefly, semiconfluent cultures were treated with 10^{-6} M

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4NQO for 1h at 37°C, and then the culture medium containing 4NQO was replaced by fresh medium without 4NQO. When the culture became confluent, they were routinely subcultured.

Karyotypic analysis. Cultured cells were exposed to colcemid at a concentration of 0.02 μg/ml for 3-4h at 37°C. After trypsinization, the cells were resuspended in a hypotonic solution (0.075M KCl). Following fixation in methanol/acetic acid (3:1) the cells were spread on clean slides. Metaphase chromosomes were banded by the trypsin-Giemsa method of Seabright (13).

Results and Discussion

To immortalize human fibroblasts, cells were exposed to single 1-h treatments with 4NQO from primary culture to passage 51. The immortalization process is illustrated in Fig. 1 and is described in detail by Bai *et al.* (7).

The present results support previous studies (7, 14) which showed that without repeated treatments with mutagens, human cells were not immortalized. This indicates that immortalization itself is a multi-step process involving several mutational genetic events. Support for this idea exists in the finding of four complementation

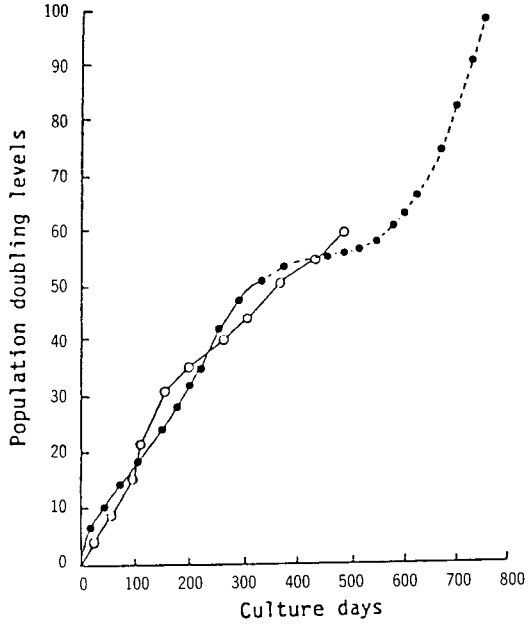

Fig. 1 Cumulative doubling levels of control and immortalized cells. The cells were repeatedly treated with 4-nitroquinoline 1-oxide (4NQO) during the primary culture to passage 51. (○): control; (●): immortalized cells.

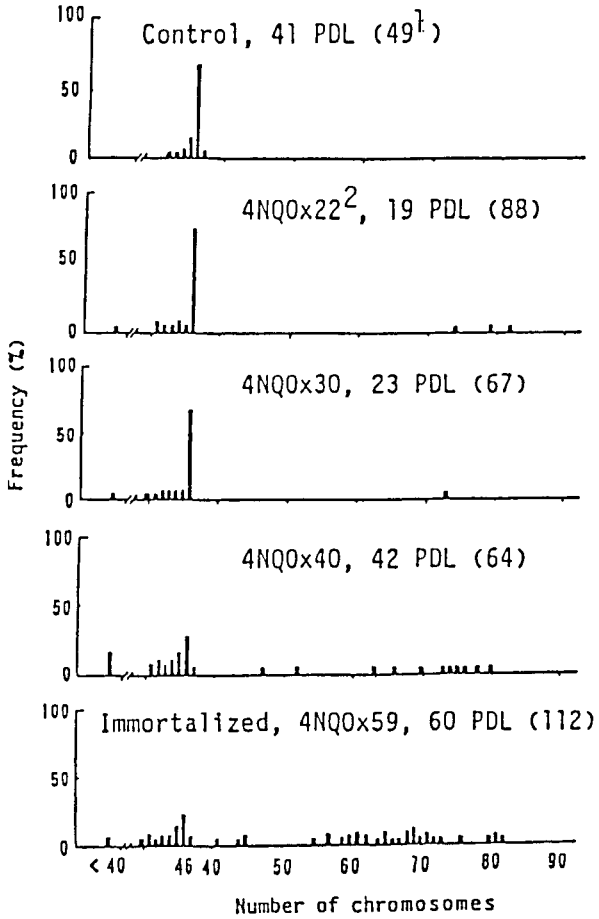

Fig. 2 Chromosome distribution 4NQO-treated and immortalized cells. [1]Number of metaphases analyzed; [2]Number of 4NQO treatments; PDL: Population doubling level. 4NQO: See Fig. 1.

groups associated with the cellular aging or immortalization of human cells (11).

In light of these findings which indicate that genetic alterations may be responsible for immortalization of human cells, we decided to examine chromosome changes during the course of immortalization of human cells treated with 4NQO. The parent cells had a normal diploid karyotype of 46 XX. As shown in Fig. 2, the number of cells having hypodiploid chromosomes increased as the treatment progressed until the 23rd population doubling level (PDL). By contrast, some cells showed hyperdiploid. The increased chromosomes in these cells were chromosomes 6, 16, 17, 18, 19, 20, 21, 22, X and others which could not be identified.

Norwood *et al.* (10) reported that when proliferating cells in early passages were fused with non-proliferative

Table 1 Karyotypic analysis for human fibroblasts during the immortalization process by treatment with 4-nitroquinoline 1-oxide (4NQO)

PDL[a]	4NQO	Meta-phase analyzed	Distribution of chromosome			Number of chromosomal loss: Chromosome number																						
			<2n	2n	2n>	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
19	22[b]	20	10	10	0			1	1	1			1		1	2	1	1		3					3	2	2	2
23	30	25	22	3	0	1	2	2	1		4	3	8	3	5	2	1		2	6	1	1	1	3	3	2	2	1
42	40	20	17	1	2	1	3			2	3	1	5	1	5	2	3	1		7		4	4	4	1			3
51	56	20	9	1	10		3			1	1		2			2	2			4		1		3	2		1	
			Total number			2	8	3	2	4	8	4	16	4	11	8	7	2	2	20	1	6	5	10	9	4	5	6

a: Population doubling level. *b*: Number of 4NQO treatments.

aging cells, DNA synthesis of the replicative cells ceased. This demonstrates that the phenotype of cellular aging is dominantly expressed and that cell immortalization may be due to the absence of cellular aging factors which lead to the deregulation of genes associated with cell growth. If this is the case, the loss of the chromosomes which contain the aging genes should induce immortalization of cells. As shown in Table 1, although relatively random loss of chromosomes was observed before immortalization of cells treated with 4NQO, chromosomes 2, 6, 8, 10, 11, 12, 15, 19, and 20, were lost at a significantly higher frequency. On the other hand, as mentioned above, the increase of chromosomes was random and without any significant high frequency. Thus, it was impossible to determine from the present data which chromosomes, if any, must be increased for immortalization of human cells to occur.

There have been many reports which relate chromosome losses or changes to immortalization of human cells by SV40. Naiman and Canaani (15) reported that human fibroblasts treated with SV40 DNA lost chromosomes 19 and 22 prior to immortalization. Goolsby *et al.* (16) observed that the only consistent chromosome rearrangement before immortalization of human fibroblasts treated with SV40 DNA was t (3:?) (q27:?). Just after immortalization they observed t (2:4), t (2:14), t (3:?), del (6) (p11), i (6p), del (8) (p12), t (14:14), i (15), and t (18:?). Hoffschir *et al.* (17) reported losses of 11p and 6q in seven SV40-immortalized human cell lines. Ray and Kraemer (18) described three or more of the following minimal chromosomal deletions specifically associated with the immortalization event: del (6) (q21), del (3) (p24), del (1) (p34), del (4) (p25), del (5) (p14), del (11) (p11), del (11) (q14), del (12) (p12), and del (14) (p?). Hubbard-Smith *et al.* (19) demonstrated that the SV40-immortalized cell lines had loss of chromosome 6, especially the portion distal to 6q21. An explanation for why the chromosomal changes in the SV40-immortalized cells mentioned above were not extensive could be that products of SV40 inactivate so-called tumor suppressor genes such as Rb and p53 by binding Rb and p53 proteins. In comparison to SV40-transformed cells, our 4NQO-treated cells showed prominent chromosomal aberrations which might be attributable to repeated treatments with 4NQO, a very potent mutagen. However, only repeated extensive treatments were able to immortalize human cells. This indicates that the immortalization process itself could be comprised of multiple mutational events. In any case, all the above-mentioned data indicate that losses of some chromosomes always occur and seem to be critical in the immortalization process of human cells. Ning *et al.* (12) provided evidence that loss of chromosome 4 is needed for immortalization of human cells. They introduced normal human chromosome 4 into immortalized cells and observed that the cells reverted to normal cellular aging patterns. Recently, similar results have been obtained on chromosomes 1, 6 and 7 (20–22); these results indicate that the recessive immortalization phenotype may result from a limited number of specific genetic alterations rather than from random events. We believe that it may soon be possible to identify this limited number of specific chromosomes that closely related to immortalization.

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