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NATURAL AND CONDITIONAL AGEING OF MOUSE FIBROBLASTS: GENETIC VS. EPIGENETIC CONTROL

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SUMMARY: Mouse embryonic fibroblasts were fused with a spontaneously immortalized mouse fibroblast cell line and a large number of heterogeneous hybrid populations were obtained. These showed variations with respect to their chromosomal number as well as *in vitro* life span but none aquired immortal phenotype. Apart from demonstrating the dominant nature of senescence over immortalization in mouse system, we also provide the first report on the analysis of genomic DNA methylation during *in vitro* passaging of parental and hybrid cell populations representing the normal and conditional ageing, respectively. Since no random decline in DNA methylation could be detected in any of the cases, our results suggest that it is unlikely that mortality of cells in culture is the outcome of random loss of epigenetic control imposed by 5-methyldeoxycytidine at CpG sites in the genome.

Multiple attempts have been made to trace the molecular basis of fixed mortality of cells in culture following its first description by Hayflick and Moorhead (1). They demonstrated that the finite division potential of human diploid fibroblasts is determined by the physiological rather than the chronological age of cells. The mechanism by which this phenomenon takes place is still unknown. Numerous hypotheses have been put forward to explain cellular senescence and its relation to *in vivo* ageing. Study of fixed mortality of cells in culture in conjunction with the rare and recessive event such as immortalization demands its genetic basis (2). However, another school of thoughts focuses on to the stochastic errors gradually accumulated at different levels. One such candidate is the gradual loss of epigenetic control of transcription imposed by 5-methyldeoxycytidine (5mC) (3,4). The latter has been demonstrated to be efficient gene silencing mechanism (5,6). Since the tissue specific methylation patterns have also been implicated in many of the basic processes such as embryogenesis, differentiation and carcinogenesis (7,8,9); it is rather tempting to think that gradual loss of DNA 5mC results into metabolic perturbations and

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deleterious phenotypic effects associated with senescence. We have exploited the availability of spontaneously immortalized cell lines of mouse fibroblast origin to test the contribution of genetic and epigenetic control to senescence. The hybrids obtained by the fusion of mouse embryonic fibroblasts (MEF) with MN48-1 (NIH/3T3 derivative) were studied for their cumulative division potential. We demonstrate the dominant nature of mortality in a large number of karyotypically heterogenous hybrid cell populations. The latter constituted the forced mortal situation of our system and was analysed for their 5mC content during serial cultivation along with the parent cells to know the involvement of epigenetic control. We report that the immortal cell line as well as the mortal cells (parent MEF and their hybrids with spontaneous immortal cell line) sustain stable levels of methylation throughout their serial passaging.

MATERIALS AND METHODS

Cell Culture: Mouse embryonic fibroblast (MEF) cultures were initiated from 17 day old CD1-ICR, decapitated and degutted mouse embryos. The embryonic skin was washed twice with phosphate-buffered saline (PBS), pH 7.2, sheared by passing through 10 ml syringe and digested twice by stirring gently in 0.5% trypsin-EDTA solution for 15 min each. The filtrate obtained through sterile musline cloth was mixed (1:1; v/v) with growth medium, Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (Biocell, USA), penicillin, streptomycin (Sigma, USA) and fungizone (Gibco, USA), and seeded at 5 X 10^6 cells/150-mm dish. The medium was changed 4-5 times during the next 2 days and finally aliquots of healthy fibroblast population were frozen at -70 °C. The cultures were routinely maintained on growth medium incubated at 37 °C in humidified atmosphere of 95% air and 5% CO_2. The cells were carried to the final stage of their *in vitro* life span by 1:4 serial passaging. Population doublings (PDS) at every split time were calculated as log (N_H) - log (N_I) / log 2, where N_H = cell number at harvest; N_I = initial cell number. The MN48-1 cell line is a ouabain- and 6-thioguanine-resistant clone of NIH/3T3 cells, kindly provided by Dr. M. Noda, Cancer Inst., Tokyo. Thus cell hybrids of this cell line with MEF could specifically be selected in the presence of ouabain and HAT.

Cell Hybrids. Cell hybrids were generated by 50% polyethylene glycol-6000 aided fusion of MEF cells during exponential growth phase (MEF 4) as well as senescent phase (MEF 7) with MN48-1. The hybrids were doubly selected on selection medium containing 4.7 mM ouabain (Sigma) and 2.5 X HAT (Sigma) conferring to 50% survival of resistant and 0-2% survival of sensitive parent cells. These concentrations were selected from independent experiments using 0-10 mM ouabain and 0-5 X HAT for each of the parent cell line and by counting the percent survival as well as colony forming efficiency. The hybrid clones were isolated and serially passaged (1:4) parallel to the control cultures.

Chromosomal Analysis. Cells at 70% confluency were treated with colchicine (0.05 μg/ml) for 45 min, fixed thrice in methanol-acetic acid (3:1, v/v) after hypotonic treatment (0.075 M KCl, 10 min, 37 °C), stained with Giemsa as well as quinaqualine mustard dyes and visualized under Olympus BH2 microscope. At least 20 metaphases per slide (n = 3) were observed for chromosome number.

DNA Analysis: Cells were harvested with PBS and incubated with proteinase K (0.1 mg/ml), RNase A (0.1 mg/ml) and 0.5% sodium dodecylsulfate (SDS) in 10mM Tris

HCl/1mM EDTA, pH 8.0 at 37 °C overnight. Genomic DNA was extracted as described (10). Isoschizomer restriction enzymes *Msp* I and *Hpa* II (Toyobo, Japan) were used to detect the DNA methylation patterns of total genomic DNA. Restriction digestion was carried out by using 3.5 units of enzyme/μg DNA at 37 °C for 14-16 h and was confirmed for complete digestion on 1.2% agarose gel. pBMGneo plasmid (11) was used as a positive control for *Hpa* II activity and the digestion was carried out by using 1 unit enzyme/μg DNA for 2 h only. Restriction digests were end labelled with Klenow large fragment using [α-^{32}P]dCTP, extracted with phenol chloroform and precipitated with ethanol using *E. coli* DNA as carrier. Redissolved DNA was separated in a 1.8% agarose gel by loading 2 X 10^7 cpm/lane of the samples indicated. The gel was vacuum dried onto nylon membrane (Hybond N+, Amersham, UK), and autoradiographed. Alternately, restriction digests were transferred to nylon membrane (10) and probed with highly repetitive (GATA repeat unit) 545 bp *Drosophila* genomic clone 2(8) kindly provided by Dr. Singh, CCMB, India (12). Hybridization was performed at 65 °C overnight using 3 X 10^6 cpm of random primed probe/ml of 0.5 M phosphate buffer containing 7% SDS. After two washes in 2 X SSC-0.1% SDS the membrane was autoradiographed.

RESULTS AND DISCUSSION

In the present series of experiments, *in vitro* life span of MEF cells as calculated from (1:4) serial passaging was 30 PDS which is taken as 100% life span. No increase in cell number was detected thereafter for about 30 days, rather cells undergo crisis and death (n = 6). The generation time of the population increased from 1.1 to 4.9 days during this period. Five morphological phases could be distinguished during their *in vitro* ageing with each corresponding to the serial lapse of 20% of total life span (Fig. 1). Cells were observed for about 3 weeks after they had entered phase E. They were made sure of being mortal by the lack of division and deteriorative morphology. During late senescence periods (PDS = 24 to 27), morphologically younger looking cells were rarely observed in the background population of old non-dividing cells. However, on isolation so far, they were unable to establish. Thus under our experimental conditions such as initiation of culture from embryonic skin and subsequent maintenance on relatively less volumes of growth medium CD1-ICR MEF cells immortalize and establish with much less frequency than expected (13). The observed stability of MEF cells under our defined experimental conditions in addition to the other merits such as simplicity, short generation time, short *in vitro* life span urged us to use these cells for the present studies.

Fibroblasts at their 20% (6 PDS) and 70% (21 PDS) life span were fused with established line MN48-1. The hybrid cells were selected on HAT-ouabain medium at 50% stringent conditions established from independent experiments. Table 1 details the population doubling potential of hybrid cells obtained in two independent fusions. Cells were maintained for at least five weeks after they had stopped dividing. None of the hybrid clones could be established into permanent cell line rather the five morphological stages similar to the mortal parent (MEF) could be traced (Fig. 1) in more than 80% of the hybrid populations. Karyotypical analysis of many of the hybrids

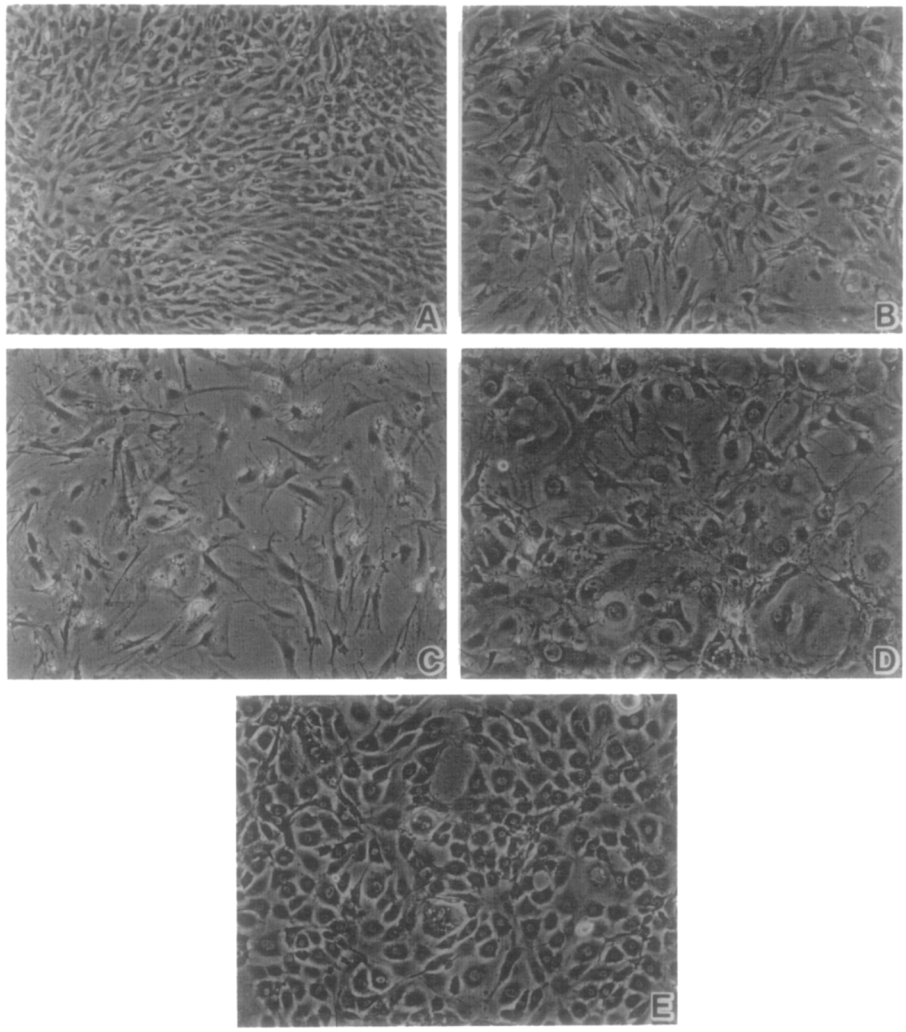

Fig. 1. Five morphological phases of MEF during *in vitro* ageing. Phase A-E correspond to 20,40,60,80 and 100% of cumulative population doublings respectively.

reveals their subtetraploid nature (Table 1). Due to the high number and acrocentric nature of chromosomes, the analysis could not be extended beyond counting the chromosomal number. The cumulative population doubling (CPDL) of hybrids were observed to be inversely related to the physiological stage, 6 or 21 PDS, of parent MEF cells at the fusion time. This strengthens the concept that fixed mortality is a genetic trait and is dominant over immortality (2). Hybrids varying from chromosome number 99 to 106 formed the main class (*Group I*) in two independent experiments and their total *in vitro* life span inclusive of the parent MEF cells ranged from 38 to 42 PDS. Hybrids with 96 to 97 (*Group II*) chromosome number had extended life span (45 to 48 PDS) whereas the *Group III* with 108 to 113 chromosome survived for 32-35 PDS. Thus the CPDL of hybrids was seen to exceed the number (30 PDS) as expected from the parent mortal cells in at least three independent experiments. This

Table 1. Cumulative population doublings (CPDL) and chromosome number (Ch. No.) of hybrids

MEF PDS-6 X MN 48-1 Hybrids			MEF PDS-21 X MN 48-1 Hybrids		
	CPDL*	Ch. No.**		CPDL*	Ch. No.**
Group I					
1. C-8	36	99	1. P-54	19	99
2. P-18	35	101	2. P-55	18	102
3. P-24	36	106	3. P-56	21	101
4. P-26	33	101	4. P-59	21	102
5. P-27	36	99	5. P-60	18	106
6. P-30	34	102	6. P-61	18	101
7. P-32	36	102	7. P-62	17	103
8. P-35	32	101	8. P-63	18	99
9. P-36	34	99	9. P-64	20	102
10. P-41	32	102	10. P-65	19	104
11. P-45	34	101	11. P-66	18	101
			12. P-67	20	99
Group II			13. P-68	18	106
1. P-16	42	96	14. P-69	18	101
2. P-42	39	96			
3. P-47	39	97			
4. P-6	42	96			
Group III					
1. P-20	29	108			
2. P-22	29	113			
3. P-37	26	109			
4. P-53	29	110			

* n=3
**corresponding to 80% populations

can be viewed as a drift imparted by immortal genotype. We suggest that the quantitative differential expression of certain factors determines the mortal or immortal phenotype. Fusion between mortal and immortal cells possibly provides the high level of such factor(s) along with conditions in which it could not be sustained (mortal being dominant). However, an initial stepping up of such factor(s) might be the cause of extended longevity. The nature of such factor(s) is an open question. Nonetheless, demonstration of the dominant nature of the mortal phenotype in a large number of heterogeneous hybrid populations obtained by the fusion of mortal and spontaneously immortalized cell line of the same origin are important observations.

The heterogeneous hybrid populations described above constituted a good system with forced mortal condition along with the parent mortal and immortal cells to study the correlation of epigenetic errors to senescence. The latter was analysed by using a pair of restriction enzymes with the same cutting site CCGG, where one (*Hpa* II) is sensitive to methylation at C residue. Gradual decrease in 5mC content, if any, would result into increase in *Hpa* II cutting sites as it has been established that practically all methylation occurs at CpG sites in eukaryotes (14). We screened for such increase in *Hpa* II sites by two independent methods, i.e., direct labelling of

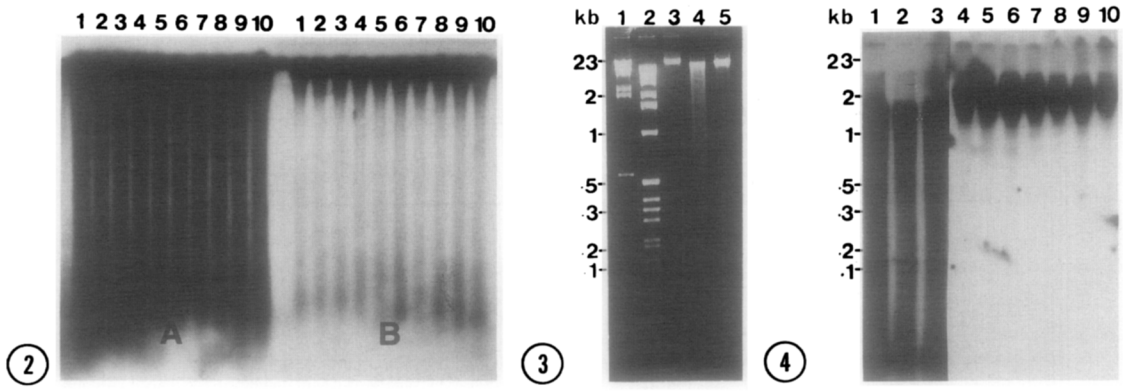

Fig. 2. [α -^{32}P]dCTP labelled *Msp* I (A) and *Hpa* II (B) restriction digests of serial passage cultures of MEF and hybrid cells (n=6) separated in a 1.8% agarose gel. Lanes 1-5 correspond to MEF phase A to E of Fig. 1 respectively and lanes 6-10 correspond to parallel phases of hybrid cells.

Fig. 3. *Msp* I (lane 4) and *Hpa* II (lane 5) digests of MEF genomic DNA (lane 3) separated with ethidium bromide in a 1.5% agarose gel. Lanes 1 and 2 are λ/*Hin* dIII digest and 1 kb DNA ladder respectively.

Fig. 4. Southern blot of ageing MEF and hybrid cells hybridized with GATA rich *Drosophila* genomic clone 2(8). Lanes 1-3 (*Msp* I) and lanes 4-6 (*Hpa* II) are restriction digests of MEF cells corresponding to phase A, C and E of Fig. 1. Lanes 7-10 are *Hpa* II digests of hybrids corresponding to morphological phases A, C, D and E of Fig. 1.

restriction digests and hybridization of unlabelled restriction digests with a highly repetitive probe (12). Both the methods are sensitive and specific enough to detect the increase of even few sites (6,12,15,18). Together with our established system, the assay offered a direct approach to access the contribution of epigenetic control to senescence. Figs. 2, 3 and 4 show *Msp* I and *Hpa* II restriction digest patterns of mortal, immortal and hybrid cell populations as analysed by different methods at indicated passage number, though the cells were studied at every passage. Restriction digest patterns revealed that the majority of CpG sites are methylated in mouse genome as the repetitive probe 2(8) could pick up only high molecular weight bands on *Hpa* II digests with almost complete absence of the smear detectable on *Msp* I digests. Direct labelling of restriction digests showed the same result. As is clear from the serial analyses the expected random increase in *Hpa* II sites was not detectable at any stage of *in vitro* ageing of parent as well as hybrid cell populations. The analysis was performed on three independently serially passaged MEF cultures and at least 10 of the hybrid clones showing serial ageing parallel to MEF cells. Immortal cells MN48-1 were also analysed five times at the gap of 10 splittings each. None of the ageing cell populations showed a loss of DNA methylation during serial passage analysed till terminal stages. Epigenetic errors through loss of methyl groups have been proposed to contribute to the ageing process (4). This has been supported by reports such as, correlation of rate of loss of methylation in mice, hamster and human cells to their *in vitro* CPDL (16); 5-azacytidine induced life span

shortening of MRC-5 cells (3,17) and high levels of methylation in some permanent lines (18). However, loss of methyl groups has not been related to the number of division in culture (16). Instead, different tissues such as brain, liver and small intestinal mucosa have been reported to have similar rates of 5mC loss in spite of the fact that they age very differently and have widely varying physiological functions and mitotic index (19). Tissues and cell populations from 8 months to 82 years old humans are reported to have highly variable 5mC content and there is no relation to cell renewal and age (20). Thus the contribution of epigenetic errors to ageing process remains controversial so far. It seems that there has been the lack of proper system to access the relationship of 5mC content to the Hayflick limit of cells. We have analysed 5mC levels under natural and conditional mortal situations of the same system. Our approach could have potentially delineated the relation, if any, of such random epigenetic errors to senescence. Since we could not detect any decline in methylation during serial passaging of our system, we think that fixed mortality of cells can not be attributable to the random loss of epigenetic control such as gradual demethylation of CpG sites.

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