Indian Journal of Science and Technology http://www.indjst.org Vol.1 No.1 (Nov. 2007)

Comet assay to monitor cell line aging

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Abstract: 'Comet assay' becomes a powerful tool in bioassay and cell lines are the preferred study-systems in the light of stringent bioethic norms. Human B lymphocyte-derived cell lines at different passage levels were subjected to alkaline comet assay. Cell lines at higher passages exhibited increased DNA damage compared to that of lesser passages. In addition, apoptotic conditions were also found more at higher passages. When cell lines were subjected to irradiation (2 Gy of ^{60}Co-γ), the tailmoment generated as a result of the radiation-insult was found to be influenced by passage factor. Thus 'passage factor' can impose for appropriate correction measures when cell lines are used in genotoxic test. Single cell gel electrophoresis can be a simple method to assess the suitability of a given cell line for toxicological studies.

Keywords: Cell line, Comet assay, Aging, Lymphocytes, DNA repair, Radiation

Introduction

In the light of stringent bioethic norms, cell lines and tissue cultures are increasingly used as model systems in understanding the response of an organism to various physico-chemical- factors. In recent investigations, single cell gel electrophoresis or 'comet assay' has emerged as a powerful technique to probe such a response (Anderson *et al.,* 1998; Heaton *et al.,* 2002). It is a sensitive and rapid technique to visualize individual cells from a heterogeneous population for their various levels of DNA damages, apoptotic and necrotic states (Olive,1999). Comet assay has been applied successfully to study heterogeneity of senescent populations and recorded DNA damage in senescent fibroblasts (MRC5 human fibroblasts) (Mocali *et al*., 2005). The alkaline-comet assay is the most predominantly used method wherein DNA strand breaks, regardless of their origin, and other lesions yielding alkali-labile sites can be easily detected. 'Tailmoment' is considered as the most reliable parameter in this assay to express the degree of genotoxicity. But mammalian cell culture of different types has a finite proliferate potential with wide variation of *in vitro* lifespan (Hayflick, 1965), apparently linked with cellular aging. DNA-damage is commonly evident in aging cells (Williams & Dearfield, 1981). The variety of DNA-lesions reportedly associated with aging includes single strand breaks and chromosome aberrations. In this paper it was investigated whether the cellular changes associated with cell line aging can influence the 'tailmoment'. For this purpose human lymphocyte-derived cell line (T14) was subjected to comet assay. Ionizing radiation was used as genotoxic agent to study its response at different passages. The behaviour of those cell lines that carry radiation-insult after different passages (T8 and T13) was also studied. Freshly prepared lymphocytes were used for comparison.

Materials and methods

Chemicals:

The chemicals used in this study were purchased from the following suppliers: agarose and low melting agarose (LMP) from Gibco BRL (Gaithersburg, USA); EDTA, NaOH, and triton X-100 from Merck (Darmstadt, Germany); dimethyl sulfoxide, NaCl, propidium iodide, Tris and RPMI 1640 medium from Sigma Chemicals (St. Louis MO, USA).

Establishment of lymphocyte cell lines:

Lymphocyte B-cell lines (T14, T8 and T13), established at the same time from same male donor in this laboratory, were used. For cell line establishment, about $2x10^7$ cells of lymphocytes from normal adult, separated by Ficoll-sedimentation, were washed twice with PBS solution and infected by Epstein-Barr (EB) virus for 12 h. The cells were cultured in RPMI 1640 medium containing 20% fetal bovine serum at 37 ^{0}C for a week. The 5 ml of the cell suspension were transferred to 76-well microplate and grown for several months. The growing colonies derived from a single cell were transferred on 4-well microplate and cultured for a month for expansion. The colonies were further separated and cultured as T14 cell-line in 50 ml culture flask to obtain $2x10^7$ cells for studies. T8 and T13 cell-lines were obtained by the same method but the lymphocytes were irradiated with 0.125 Gy of ^{241}Am-α (0.018 Gy/ sec; Radiation Biology

Centre, Kyoto Univ.) and 0.5 Gy of ^{60}Co-γ (0.02 Gy/min; RIRBM, Hiroshima Univ.) respectively, prior to EB virus infection. Cell-lines were passaged once in a week.

Repair study :

Cell-lines at the final titer of 10^7/ml were irradiated to 2 Gy of ^{60}Co-γ rays (0.1 Gy/min) at 37 ^{0}C and incubated for 1 h before performing comet assay. Control samples received sham treatment.

Single-cell gel electrophoresis:

The basic alkaline technique described by Singh *et al*. (1988) was followed with some modifications (Gajendiran *et al*., 2000). About 10^4 cells in 5-7 µl were mixed with 80 µl of 0.7% low-melting agarose at 37^{0}C and spread on a fully frosted microscopic slide pre-coated with 200 µl of 1% agarose. Oncor Chromosome In -situ System plastic cover slip (Oncor Science Inc., USA) was laid over the gel mixture to get uniform surface. After gelling at 0 ^{0}C for a minute, the cover slip was gently pealed off from the agarose layer. The cells were lysed by dipping the slides in a lysing solution (100 mM Na-EDTA, 10 mM Tris, 2.5 M NaCl, 1% Triton X-100 and 10% DMSO, pH 10) for 1 h at 4 ^{0}C to remove membrane and proteins. The slides were rinsed free of salt and detergent in buffer (1 mM Na-EDTA, 300 mM NaOH, pH>13) and subsequently submersed in a horizontal gel electrophoresis apparatus by adding fresh buffer and remained in the buffer for 20 min to allow unwinding of DNA and expression of alkali labile damage. Then, a weak electric field was applied (300 mA; 0.8 V/cm for 20 min at 24 ^{0}C, under dim yellow light) to draw negatively charged DNA towards anode. After electrophoresis, slides were washed twice for 5 min in neutralizing buffer (0.4 M Tris, pH 7.5) and stained with 75 µl of propidium iodide (20 µl/ml). Slides were stored in a moist chamber at 5 ^{0}C and used for analysis within 3 h. Under this condition, each cell can be identified with a landmark of migrating DNA. The amount and length of migration greatly depends on the DNA fragment size. The experiments were repeated at least thrice and the average value was used in graphic representation.

Comet image analysis:

The fluorescent signals of the stained 'comets' were examined using a fluorescence microscope with excitation at 530-560 nm, detection >580 nm, coupled with an intensified target camera with a self-designated image analysis system (Olympus, Tokyo). The comet images were stored using CCD camera and analyzed using software in BAS 1500 (Fuji Co., Tokyo). In the first step of the measurement, the head of each comet was marked by a circle, followed by measurement of DNA content within this circle. Finally, the total fluorescence of head and tail was calculated by subtracting the head fluorescence from the total fluorescence. The genetic integrity of the heterogeneous cell population was understood as tailmoment in individual comet cell. The tailmoment was calculated by multiplication of the tail length by the amount of DNA in the tail. The comet tail was set to be the area from the edge of the head to the end of the tail. Relative units were used in the graphic presentations. Apoptotic cells were separately counted. They can be distinguished from the remaining cells by its very appearance, as its DNA is extremely fragmented and able to migrate completely away from the head position. Statistical analyses were carried out using the INSTAT GRAPH-PAD program. ANOVA was employed to compare significance among means and ±SD values.

Results and discussion

Cell-lines at higher passages showed enhanced tailmoment than the lesser passages or freshly prepared lymphocytes (Fig. 1; Table 1 & 2). In late passaged cells, the decrease in fidelity of DNA polymerase α and proof reading activity must lead to mutations in DNA (Fukuda,1999). Mutations appear to increase as a function of age. Lethal mutation may lead to apoptosis (Mothersill, 1996). The higher number of apoptotic cells was found associated with high passage levels (Table 3).

Table 1: The outcome of 'tailmoment' in irradiated and unirradiated cell samples at different 'passage' levels

Sample	Tailmoment*		
	Unirradiated	Irradiated (2 Gy ^{60}Co γ)	
		'0' h	After 1 h Repair
C	12.6 ± 4.1	156 ± 41	101 ± 32
T8 (1)	34.2 ± 13	246 ± 86.1	214 ± 93.2
T8 (33)	78 ± 23	380 ± 111	351 ± 144
T13 (1)	25.6 ± 8.1	178 ± 71.9	134 ± 40
T13 (34)	74.5 ± 21.7	347 ± 110	307 ± 106
T14 (1)	23.1 ± 6	164 ± 67	122 ± 40
T14 (48)	40.7 ± 8	389 ± 110.8	341 ± 109

No: in parentheses indicates passage level
**Values are represented in mean and ±S.D. of the mean; C' is a freshly prepared human lymphocyte sample; 'T8, T13 & T14 are its cell line*

The cell lines when subjected to other end point analysis, T8 and T13 had the highest chromosomal instability with dicentrics and rings, chromatid type aberrations, hyperdiploid, endoreduplication and telomere-fusion, at higher passages (unpublished data). Thus, the diversity of DNA changes found in aging cells often correlates with growth variations. Cellular aging occurs both *in vitro* and *in vivo* and the accumulation of persistent cellular damage consistent with age has been indicated (Chow & Rubin, 1996).

of increasing tailmoment associated with the 'passage' number of the cell lines, particularly over freshly prepared lymphocytes of their own origin, emphasizes the need to consider the 'passage' factor while making comparison. Comet assay can be a rapid method to assess the genetic integrity of the given cell line of unknown passage.

Table 2: Statistical comparison of the response of cell lines subjected to comet

Sample	Unirradiated			Irradiated (2 Gy ^{60}Co γ)					
	t	*P*	*S/NS*	'0' h			After 1 h Repair		
				t	*P*	*S/NS*	*t*	*P*	*S/NS*
C vs T8 (1)	2.745	0.0517	NS	1.635	0.1775	NS	1.931	0.1257	NS
C vs T8 (33)	4.849	0.0083	S	3.279	0.0305	S	2.898	0.0442	S
T8 (1) vs T8 (33)	2.871	0.0454	S	1.652	0.1738	NS	1.383	0.2387	NS
C vs T13 (1)	2.48	0.0682	NS	0.4604	0.6692	S	1.009	0.370	NS
C vs T13 (34)	4.855	0.0083	S	2.818	0.0479	S	3.172	0.0338	S
T13 (1) vs T13 (34)	3.657	0.0216	S	2.227	0.0899	NS	2.645	0.0573	NS
C vs T14 (1)	2.503	0.0666	NS	0.1764	0.8685	NS	1.996	0.1167	NS
C vs T14 (48)	5.414	0.0056	S	3.416	0.0269	S	3.61	0.0226	S
T14 (1) vs T14 (48)	3.048	0.0381	S	3.01	0.0396	S	2.841	0.0468	S

"t" represents the value with four degrees of freedom; "p" represents the two-tailed p value; "S" represents the statistically significant value, while "NS" means non-significant
Refer Table 1 for legend.

The radioresponse of the cell lines at different passage levels was also studied. When cell lines were subjected to irradiation (2 Gy of ^{60}Co-γ), the tailmoment generated as a result of the radiation-insult was found to be influenced by passage factor. The enhancement of tailmoment was higher in T13 and T8 cell-lines than that of T14 and the isolated lymphocytes. Also T13 and T8 cell-lines exhibited lesser repair than the others, but the level of repair was independent of the passage factor (Fig.2). The exact mechanism behind the above observation is not known, but the type of low dose radiation received by clonal cells during establishment of the cell line (T13 and T8) result in genetic instability and probably owes for their higher tailmoment compared to T14 and freshly prepared cell line. Thus, apart from aging, the genetic integrity of the clonal cells during cell line establishment seems to influence the outcome of 'tailmoment' after several passages. Generally, cell lines of high passage level responded with significantly higher tailmoment compared to freshly prepared lymphocytes (Table 2 & 3). The trend

*Table 3: Apoptotic cell population **

Cell-line	0 h	After 1 h incubation
C	2 ± 1	20 ± 4
T8 (1)	10 ± 2	30 ± 9
T8 (33)	40 ± 4	50 ± 13
T13 (1)	2 ± 1	30 ± 5
T13 (34)	30 ± 10	40 ± 10
T14 (1)	2 ± 1	20 ± 6
T14 (48)	20 ± 4	40 ± 8

**in 1,000 comet cell population*
Refer Table 1& 2 for legend

Conclusion

Cell lines of different passage levels differ by tailmoment parameter. The alkaline single cell gel electrophoresis forms a docile method to monitor senescence-linked DNA damage in cell lines of human origin.

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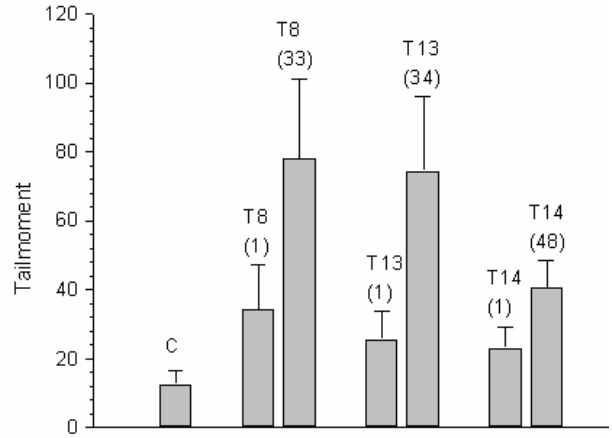
Acknowledgements

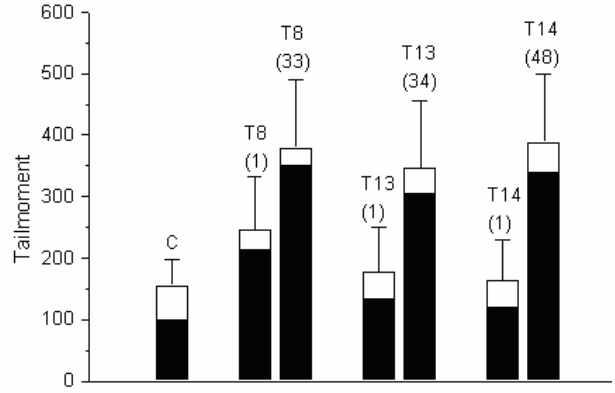
NG thanks Dr.M.S.Sasaki and Dr.Y.Ejima, Radiation biology Center, Kyoto Univ., for technical help for ɣ-ray irradiation and dosimetry. The author gratefully acknowledge the expert discussion and initial training given by Dr.T.S.Kumaravel and Dr. K.Tanaka. NG was supported by HICARE, Japan & IGCAR, Kalpakkam.

Fig.1: Influence of 'passage'-factor in comet assay



"C" is freshly prepared human peripheral blood lymphocytes; T8, T13, and T14 are cell lines derived from human peripheral blood lymphocytes; Number in parentheses indicates 'passage' level; Error bar represents ±SD of average mean.

Fig.2: Radioresponse of human lymphocyte cell lines at different passage levels



Solid bar represents tailmoment after 1 h of repair; The solid + open column represents the initial tailmoment immediately after irradiation (0 h); Refer Fig.1 for legend.