Comet Assay as an Aid to Pap Smear Test

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Summary: **Carcinoma of the cervix is preceded by dysplasia, which has been diagnosed and classified into different grades on the basis of cytomorphological Pap Smear Test. However, Pap smear test is not a foolproof test. In 5-10% of the cases the results may be either false positive or false negative. Moreover, boundaries between the grades are not sharp. Keeping in view the need to increase the efficiency of Pap smear test we used a newly developed technique, called Comet Assay, to study DNA damage in dysplasia cases. Our observations showed that not only there was a significant increase in the amount of DNA damage in the dysplasia cases as compared to the controls, there was also a significant increase in the level of DNA damage from mild to severe dysplasia. It was, therefore, concluded that in decision making, comet assay may be used as a supplement to Pap smear test in the diagnosis as well as grading of cervical dysplasia.**

Introduction

Carcinoma of the cervix is the most common cancer among women in India (WHO 1986). This carcinoma is preceded by cervical dysplasia, which may be mild, moderate or severe. However, not all cases of dysplasia progress toward cancer. It has been estimated that about 0.5% of the cases of mild dysplasia and about 10% of the cases of severe dysplasia are likely to progress to cancer (Luthra et al 1969). Therefore, for proper clinical management of a patient with cervical dysplasia, determination of the grade of dysplasia is very essential.

For determination of the grade of cervical dysplasia, the most common method used is based on the cytomorphological determination (Koss 1979). The chances of false negative report are as high as 10% and false positive 5%. There is, therefore, a need to explore the possibility of another assay which would serve as an aid to Pap Smear Test.

Most human cancers are associated with genomic instability (Heim and Mitlelman 1987). Even though the biological meaning of genomic instability on the development and progress of cancer remains unclear, high genomic instability may play a critial role in cancer predisposition or progression. Keeping this in mind, we planned to study genomic instability in terms of DNA damage in patients with cervical dysplasia. DNA damage was estimated by using a newly developed method called Single Cell Gel Electrophoresis Assay (Singh et al 1988) or commonly known as Comet Assay (Collins 1992).

Materials and Methods

The study included a total of 270 females who came to three different hospitals (Mahavir Hospital, Niloufer Hospital and MNJ Cancer Hospital)of Hyderabad for gynecological checkup which included Pap smear. Subjects were coded at the time or sampling and the data on them were decoded at the time of analysis.

a. Sample Collection :

Peripheral blood leucocytes (PBL) and cervical epithelial cells (CEC) were collected from each individual. About 100 microliters of heparinised blood was collected, by finger prick, into a microfuge tube containing PBS (Phosphate Buffered Saline). For the cervical epithelial cells, cervix was gently wiped with a sterile swab and the Ayre spatula was used to scrape the cervix in a rotatory

fashion. Two scrapings were collected from the cervix. Sample from the first scraping was spread on a glass slide and was fixed immediately in ethyl alcohol. This sample was used to study pathological status of the cervix by Pap Smear Test. Sample from the second scraping was suspended in PBS. Both the samples (PBL and CEC in PBS) were brought to the laboratory under cold conditions, and were processed within an hour of sampling. These samples were used to study DNA damage by the comet assay after checking their viability by Trypan Blue Test.

technique are as follows : (1) Nucleated cells (in this case peripheral blood containing leucocytes and cervical epithelial cells) are embedded in agarose gel on fully frosted slides. (2) The slides are left overnight in a lysing solution, containing detergents and high salt concentrations, to lyse the cell walls and nuclear membranes. (3) The slides are arranged in an electrophores is box and covered with an alkaline solution which denatures the DNA. (4) Electrophoresis is performed in the same solution Single strand breaks, if any, in the DNA produce fragments of varying length in the genome. DNA being nega

a. b.

Fig 1. a. Cell Showing comet - tail
b. Cell without a tail

b. Pap Smear Test :

The use of Papancicolaou stain resulted in well stained nuclear chromatin and differential cytoplasmic counterstaining. Based on the cytomorphological classification after Koss (1979), the subjects were divided into 4 categories: (1) Normal, (2) with mild dysplasia (MD), (3) with severe dysplasia (SD) and (4) with carcinoma of the cervix.

c. Trypan Blue Test :

The trypan blue test was performed on peripheral blood leucocytes and cervical epithelial cells, each suspended in PBS, to determine the percentage of viable cells for every individual.

d. Comet Assay:

The comet assay was carried out after the method described by Singh et al (1988). Salient features of the

tively charged, these fragments migrate toward anode during electrophoresis. (5) The slides are stained with a fluorescent dye, ethidium bromide, and examined under a fluorescent microscope. A normal cell having no DNA damage appears as a halo, whereas a cell having DNA damage appears as a comet (Fig.1) That is why, this method is down as Comet Assay. (6) Tail-length of a comet is measured with the help of an occular micrometer. This measurement gives an estimate of DNA damage in a cell.

Comet-tail-length was measured in 50 cells per subject, and its mean calculated. Means and variances were calculated for each of the 4 groups of subjects. Since the variances were highly heterogeneous, non-parametric Mann-Whitney-U Test was performed to evaluate the differences between groups. All comparisons were made at 5% level of significance.

Results :

Table 1 gives the number of subjects, their age ranges and cell viability percentages. A total of 13,500 peripheral blood leucocytes and an equal number of cervical epithelial cells were scored for their comet-tail-length in this study.

Table 1
Number of Subjects, Age Ranges and Cell Viability of Peripheral Blood Leucocytes and Cervical Epithelial Cells in 4 groups of Subjects Classified on the Basis of Pap Smear Test

Groups	Number of Subjects	Age Range (in years)	Cell Viability (%) Peripheral Blood Leucocytes	Cervical Epithelial Cells
Controls	50	17-70	93	95
Mild Dysplasia (MD)	77	17-52	92	90
Severe Dysplasia (SD)	91	24-70	90	85
Cervical Cancer	52	24-85	87	72

Table II
Mean Comet-Tail-Length of Peripheral Blood Leucocytes and Cervical Epithelial Cells in 4 groups of Subjects Classified on the Basis of Pap Smear Test

Groups	Comet Tail-Length (m) Peripheral Blood Leucocytes	Cervical Eplithelial Cells
	Mean±SE	Mean±SE
Controls	0.87±0.09	0.97±0.11
Mild Dysplasia (MD)	2.01±0.10	3.03±0.13
Severe Dysplasia (SD)	3.45±0.11	4.81±0.14
Cervical Cancer	6.52±0.27	7.54±0.26

Mean comet-tail-length of peripheral blood leucocytes and cervical epithelial cells in 4 groups, based on Pap smear test, are given in Table II. There was a significant stepwise increase in the mean DNA damage in the cervical epithelial cells as well as peripheral blood leucocytes from the normal controls to the patients with MD, SD and cancer.

Discussion

The DNA damage, as estimated by the tail-length in comet assay, was somewhat different in the cervical epithelial cells as compared to the leucocytes. Differences in DNA damage from one organ to another have also been observed in mice and humans using the comet assay (Tice et al 1990).

The general, the DNA damage was greater in cervical epithelial cells than in the leucocytes. There are four possible causes for this increased DNA damage in cervical epithelial cells :

(1) It is the target tissue for dysplasia as well as carcinoma of the cervix. (2) It is difficult to maintain hygienic conditions in the cervix because of its relative inaccessibility. (3) From the male partner the cervix may be exposed to smegma, a mutagenic substances. (4) The cervix, is subject to constant menstrual and sexual trauma, in addition to physical trauma during childbirth (Jaiswal et al 1994).

Most human cancers are associated with genomic instability (Heim and Mittelman 1987). Even though the biologial meaning of genomic instability in the development and progression of cancer remains unclear, high genomic instability may play a critical role in cancer predisposition as well as progression (Ahuja et al 1996).

There was a stepwise significant increase in the mean DNA damage from normal subjects to the patients with MD, SD and cancer (Table 2). In agreement with our observations, Murty et al (1985) have reported a significant stepwise increase in chromosomal damage in the cultured lymphocytes of patients with pre-cancerous and cancerous lesions of the cervix uteri, as compared to the controls. However, in the chromosomal damage assay among their patients with different grades of pre-cancerous lesions they were not able to show any difference, which we were able to show in the comet assay. In other words, comet assay proved to be more sensitive than

chromosomal aberration assay in differentiating between the grades of cervical dysplasia.

No doubt, Pap smear test is a classical method used for the diagnosis of cervical dysplasia, it gives about 10% false negative and 5% false positive results (Ratnam et al 1985). Pap smear results are considered false negative when clinical signs and symptoms indicate otherwise. This may be due to a deep seated growth with unaffected exfoliated cells. This type of growth may lead to clinical symptoms like a white discharge, contact bleeding, erosion, foul smelling discharge, ulceration, necrosis, inflammation or an infection (eg, that of trichomonas, fungus, staplylococcus, gonococcus or chlamydia). Similarly, in some cases this test gives a positive result when it is not dysplasia in the real sense; the cervical epithelial cells resemble dysplastic cells due to other reasons like plysiological or hormonal disturbances. Obviously, there is a need for a second diagnostic tool in false negative and false positive cases. In such cases, comet assay, which is simple and inexpensive, would come quite handy.

Pap smear test is also used for the classification of different grades of cervical dysplasia. However, demarcation between one grade to the next is not very sharp. In boderline cases, once again, comet assay may be useful in deciding the grade of dysplasia.

It is not intended here to suggest comet assay as a replacement for Pap smear test for the diagnosis and differentiation of grades of cervical dysplasia, but it may be used as an aid to Pap smear test to facilitate its efficiency. Moreover, it may not be necessary to use cervical epithelial cells in the comet assay for such an exigency; a tiny drop of peripheral blood will do the job.

Finally, the comet assay is quite versatile. There are many advantages of using this assay. It can be conducted on extremely small number of nucleated cells (ie., a few ul of blood is sufficient) and the results are obtained within a relatively short time after sampling (ie., within a few hours). It offers a high degree of sensitivity and data are obtained at the level of individual cells. Since whole blood can be successfully used, this obviates the necessity to isolate lymphocytes (Singh 1996). Although introduced relatively recently (ie. 1988) the comet assay has become quite popular around the world.

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