The usefulness of comet assay in predicting hematopoietic disorders such as myelodysplastic syndrome and acute myeloid leukemia

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Abstract: The usefulness of ‘comet assay’ in predicting the hematopoietic disorders such as MDS and AML is reported. Bone marrow biopsies of de novo AML and MDS (3 from each) were subjected to alkaline comet assay along with suitable control. The results are based on the average tailmoment of about hundred randomly selected cells. Blood malignant samples showed significantly higher mean tailmoment (AML: 195±41.6 & MDS: 501±136.2) than the normal (29.66±6.1). In addition, the spontaneous occurrence of apoptotic cells was much higher in MDS (24.5% ±5); while in AML it was <1%. ‘Comet assay’ offers as single-step visual method to predict the hematopoietic neoplasia in suspected samples.

Keywords: myelodysplastic syndrome, acute myeloid leukemia, comet assay, apoptosis.

Introduction

The myelodysplastic syndromes (MDS) are common hematological malignancies usually associated with chronic cytopenias which gradually worsen due to progressive bone marrow failure or transformation into acute myeloid leukemia (AML) (Aul et al., 1998). Most patients of MDS and AML show a consistent increase in cytogenetic abnormalities than patients with non-malignant diseases (Yashige et al., 1999; Xu et al., 1998; Haase et al., 1997) but none are specific. Mild forms are often difficult to diagnose and advanced diseases are frequently associated with multiple anomalies (Plata et al., 1999). To evaluate the prognostic factors and improve the risk analysis, it is currently accepted to combine the clinical, pathological, cytogenetic and molecular data. The events associated with disease progression from MDS to AML are not clearly understood. At the same time, AML is known to be refractory disease and less prognostic condition. Hence, there is a need to differentiate this hematopoietic neoplasia for better disease management. Recently, magnetic resonance imaging technique has been applied in assessment of AML and MDS (Takagi et al., 1999) but with limitations.

Materials and Methods

Patients and sample collection

Patients with the untreated MDS and de novo AML, 3 from each group, participated in this study and were diagnosed according to French-American-British (FAB) classification (Bennet et al., 1982). MDS consisted 2 refractory anemia (RA) and one RA with excess of blasts (RAEB). AML consisted one FAB M1 and two were FAB M4. Bone marrow biopsies were taken from the patients (aged between 27-44) at the time of initial diagnosis, before treatment, after informed consent. Marrow cells from three healthy volunteers of matching age group were used for comparison.

Single-cell gel electrophoresis (comet assay)

The basic alkaline technique described by Singh et al. (1988) was followed with some modifications (Gajendiran et al., 2000). About 10⁴ cells in 7-5 µl of bone marrow (BM) biopsies were mixed with 80 µl of 0.7% low-melting agarose (Gibco BRL, Germany) at 37°C in a microfuge and spread on a fully frosted microscopic slide pre-coated with 200 µl of 0.1% agarose. Oncor Chromosome In-situ System plastic cover slip (Oncor Science Inc., USA) was laid over the gel mixture to get uniform surface for microscopic observation. After gelling at 4°C for a minute, the cover slip was gently peeled off from the agarose layer. The cells were lysed by dipping the slides in a lysis 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°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

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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, Japan). The comet images were stored using CCD camera and analyzed using software in BAS 1500 (Fuji Co., Tokyo, Japan). 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. Statistical analyses were carried out using the INSTAT GRAPH-PAD program. ANOVA was employed to compare significance among means and ±SD values.

Results

The displacement of genetic material from the cell nucleus during alkaline single cell gel electrophoresis indicated the disease pathology (damaged DNA). Blood malignant samples showed higher mean tailmoment (AML: 195± 41.6 and MDS: 501±136.2) than the normal (29.66±6.1) with P value of 0.0012, considered to be very significant (Fig.1). Comet assay is able to record the spontaneous occurrence of apoptotic cells in BM biopsies of MDS and AML in unstimulated condition. As apoptosis results from extensive fragmentation of DNA, almost all the DNA is able to migrate. The apoptotic index was higher in MDS samples (24.5 ±5%) compared to normal or AML (P<0.0001 considered extremely significant) (Fig. 2).

Discussion

The morphological boundary of bone marrow cells within MDS types and between MDS and AML is not distinct. Complex methodologies are sought to make understandings. For instance, percentage of blast cells is used as index for classification of RA, RAEB, RAEB-t and AML. But, the simultaneous analysis based on FISH and bone marrow morphology in RAEB and RAEB-t patients showed that some apparently normal bone marrow cells had leukemic signals (our unpublished observation). On the other hand, ‘comet assay’ offers as single-step visual method to predict the hematopoietic neoplasia. An important pathogenic mechanism in MDS is the premature intramedullary cell death via extreme apoptosis, explaining the apparent paradox of cellular marrow in combination with peripheral cytopenias (ineffective hematopoiesis) (Aul et al., 1998). Deletion of the long arm of chromosome 5 [del(5q)] or loss of a whole chromosome 5 (−5) is a common finding, arising de novo in 10% of patients with myelodysplastic syndromes (MDS) or acute myeloid leukemia (AML) and in 40% of patients with therapy-related MDS or AML (Herry et al., 2007). The mechanism behind the apoptosis is not fully understood. Mundle et al. (1999) determined apoptotic index using in situ end labeling of fragmented DNA. It was significantly higher in MDS cells compared to normal and the index was least in transformed-AML cells. It is of interest to note that similar pattern was observed by us with de novo AML and MDS, in this study. Apoptosis has been linked with 'good' prognosis MDS patients and a longer period for transformation to AML (Tsopliou et al., 1999). In addition, apoptosis plays an important role for eradicating leukemic cells (Bai et al., 1999). The expression of anti-apoptotic oncoproteins such as bcl-2 is probably responsible for lesser apoptotic index in AML (Bincoletto et al., 1999). Comet assay is an attractive technique for apoptotic cell measurement as it requires minimum quantity of sample (~5 µl) and one can analyze it rapidly (<2-3 h) using simple facility. It can be a useful alternative method to flow cytometry approach (Olive, 1999). Investigating the apoptotic events in MDS and AML may
improve our understanding of the disease pathology and design drug modalities.

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References
Fig. 1. Application of comet assay in analyzing blood malignancy

Fig. 2. Apoptotic cell population in AML and MDS sample

Inserts represent apoptotic and normal cells under 'comet assay'