Visualization of Numerical Centrosomal Abnormalities by Immunofluorescent Staining

Vizualizace numerických centrozomových abnormalit imunofluorescenčním barvením

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Summary
The presence of multiple centrosomes in tumor cells is associated with the formation of multipolar mitotic spindles and results in aneuploidy of both daughter cells. Centrosome amplification is a feature of all cancer cells. We have previously described centrosome amplification in abnormal B cells. Further studies of centrosome amplification in different stages of B lineage development could provide important information about multiple myeloma pathogenesis.

Key words
multiple myeloma – B cells – centrosome amplification – plasma cells

Souhrn
Přítomnost několika centrozomů v nádorových buňkách je spojena s formováním multipolárních mitotických vřetének a vede k aneuploidii dceřiných buněk. Centrozomové amplifikace jsou znakem všech nádorových buněk. Nedávno jsme popsali centrozomové amplifikace v abnormálních B buňkách. Další studium centrozomové amplifikace v různých stadiích vývoje B buněk by mohlo objasnit nové informace důležité pro patogenezi mnohočetného myelomu.

Klíčová slova
mnohočetný myelom – B buňky – centrosomové amplifikace – plazmatické buňky

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Introduction

Centrosomes are small organelles composed of two cylindrically shaped centrioles surrounded by pericentriolar material in a normal mitotic cell. The centrosome function is to direct mitotic bipolar spindles in a process that is essential for accurate chromosome segregation during mitosis [1,2]. Centrosomes duplicate once per cell cycle, and each daughter cell receives one centrosome upon cytokinesis [3]. The presence of multiple centrosomes in tumor cells is associated with formation of multipolar mitotic spindles and faulty chromosome segregation which usually results in aneuploidy of both daughter cells [3]. In addition, centrosomes have recently come into focus as part of the network that integrates cell cycle arrest and repairs signals in response to genotoxic stress – the DNA damage response [4]. It has been well established that centrosome amplification (CA) is a distinct feature of most cancer cells [5]. Recent studies have shown the presence of CA in all stages of monoclonal gammopathies. Centrosome amplification is present in about a third of multiple myeloma (MM) cases, and it is likely that CA contributes to the accumulation of genomic abnormalities in tumor cells during disease progression [8]. Using immunofluorescent staining, we have confirmed the presence of CA in early stages of plasma cells development (abnormal B-cells) for the first time [6].

In this paper, we will describe in details immunofluorescent staining of CA in different stages of MM cell development, method possibilities, its advantages and disadvantages.

Methodology

Centrin, an integral centrosomal protein, was selected as the target for determination of centrosome copy number in B-cells and plasma cells (PCs). Immunofluorescent staining of centrin in B-cells and PCs is performed with required modification for commercially available antibodies. Bone marrow mononuclear cells (BMMC) are isolated using gradient density centrifugation on Histopaque 1077 (Sigma-Aldrich, MO, USA). Cytospin slides for immunolabeling detection of CA in PCs are prepared as follows: approximately 100,000 BMMC are placed on a slide and air dried for 24 h at room temperature. In case of PCs infiltration less than 5% in BMMC, $CD138^+$ cells are sorted directly on microscopic slides by fluorescence-activated cell sorting (FACS) using anti-CD138 fluorescence-labeled antibody (Beckman Coulter, Inc., CA, USA). Then, PCs are fixed in ice-cold methanol for 5 min at room temperature. B-cells are isolated from BMMC after $CD138^+$ cells depletion using magnetic cell separation (MACS) utilizing magnetic labeled anti-CD138 antibodies (Miltenyi Biotec, Germany). Early stages of B-cells ($CD19^+$) are sorted directly on microscope slides from $CD138^-$ cell fraction by FACS using anti-CD19 fluorescent-labeled antibody (Beckman Coulter). Cytoplasmic membrane permeabilization is done by Triton X-100 0.2% (Affymetrix/USB, UK) for 1 minute at 37°C. After that, slides are placed in PBS for 10 min using gentle agitation. To prevent non-specific binding, blocking buffer (PBS with 10% normal goat serum – Santa Cruz Biotechnology, Inc., CA, USA) is added to each slide and incubated for 20 min in the wet chamber at 37°C. After incubation, the blocking buffer is poured off by gentle agitation during 10 min. After that, 20 µl of diluted (3 : 1,000) primary antibody (Centrin 1/2 rabbit polyclonal antibody Santa Cruz Biotechnology, Inc.) is applied to each slide. The slides are incubated for 1 hr in the wet chamber at 37°C. To wash out unspecific antibody binding, slides are washed 3 times with phosphate buffered saline (PBS) for 5 min each using gentle agitation. The secondary goat anti-rabbit IgG antibody (1.5 : 1000) is applied (Santa Cruz Biotechnology, Inc.) and incubated under the same conditions for 45 min. Another step of washing is done again 3 times (PBS for 5 min, light-protected). Further immunolabeling on cytospin slides is done, using immunoglobulin light chain staining, according to the procedure described previously [7]. A cover slip is applied on all slides using mounting medium antifade without propidium iodide (PI) for plasma cells and DAPI (4',6-diamidino-2-phenylindole) for B cells.

One hundred cells are scored per each slide. Up to four centrin signals (representing four centrioles of two centrosomes) can be present in a normal cell depending on the phase of cell cycle. Thus, the presence of more than four centrin signals was chosen as a criterion for CA [8]. According to the centrin copy number, we are able to identify three cell subpopulations:

1. no centrin signal (non-CS),
2. 1–4 centrin signals (1–4 CS) or
3. more than 4 signals of centrin.

Samples with more than 11% of B cells with > 4 signals of centrin are considered CA-positive (Fig. 1). The threshold for CA positivity was calculated as the M+3SD of CA-positive cells detected in healthy donors.

Methodological Pitfalls in Multiple Myeloma Research

There are two main problems in the methodological part of MM research. Bone marrow aspirate is a mixture of abnormal cells and other cell types. Each population of interest has to be separated. The second problem is the lack of cells of interest in research material for complex patient investigation. It was optimized in our laboratory and shown that immunofluorescent staining can be carried out on cells separated and sorted by FACS directly on microscope slides. Thereby, only one thousand cells are enough. In case of PCs, there is another possibility to use cytospin slides of 100,000 BMMC with further immunoglobulin light chain staining. Thus, described method gives us a possibility to analyze numerical centrosome abnormalities by detection of supernumerary centrioles on small amount of cells using commercially available antibody.

State of the Art

CA is common in MM, occurs in early stages of malignant cell development and may represent a mechanism leading to genomic instability in MM [9]. Previously, studies described immunofluorescent staining of 3 main structural centrosome proteins, including pericentrin, γ-tubulin and centrin. Studies, based

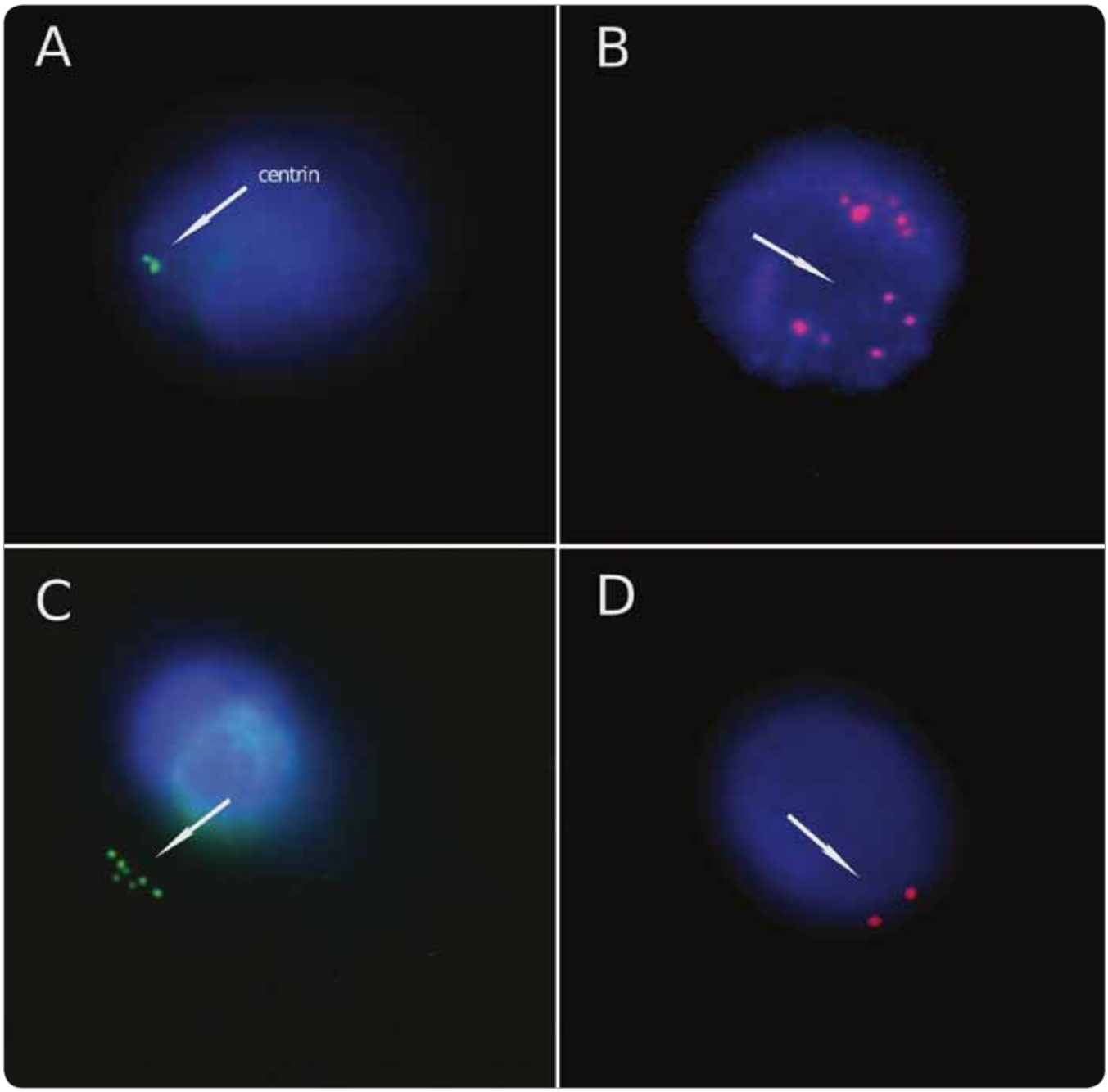

Fig. 1. B-cells (CD 19+) were identified by DAPI (blue), and centrin was stained with anticentrin 1/2 conjugated with FITC/ TR (arrow; green/red). (A, D) 2 signals – cells with 1 to 4 signals were considered to have normal centrosome. (B) Centrosome amplification (> 4 fluorescence signals of centrin) – abnormal B cell. (A, B, D) centrosomes are in projection of the cell nucleus. The cells were visualized with either Olympus BX61 fluorescence microscope equipped with a Vosskuhler 1300D digital camera and LUCIA-KARYO/FISH/CGH imaging system (Laboratory Imaging, s.r.o, Czech Republic) or microscope AXIOPLAN 2 Imaging (Carl Zeiss Imaging Solutions GmbH, München, Germany) equipped with computer analysis system ISIS (MetaSystems GmbH, Altlussheim, Germany).

on different target proteins, conclude accumulation of centrosome with disease development: the mean number of centrosomes per cell and the percentage of tumor cells with centrosome abnormalities increased progressively from MGUS to MM [10,11].

Other structural abnormalities such as increased centrosome volume, accumulation of excess pericentriolar material and inappropriate phosphorylation of centrosomal proteins are not detectable by previously described method. Supernumerary centrosomes can result from replication errors or failure of cytokinesis, whereas overexpression of centrosomal proteins, such as pericentrin, TACC and aurora kinase, can induce structural centrosomal abnormalities [12,13]. Centrosome volumes can be determined by three-dimensional rendering of confocal z-stacks labeled with γ-tu-

bulin. It was published that mean total centrosome volume highly correlates with numerical centrosome abnormalities and is significantly higher in MM compared to MGUS [10]. Study of gene expression-based index (centrosome index) comprising the expression of genes encoding the main centrosomal proteins, centrin, pericentrin, and γ-globulin, has found that it was associated with poor prognostic genetic subtypes and portends a short survival. [11]. Evaluation of expression profile of genes, involved in numerical and structural centrosome abnormalities, showed their significant increase in CA positive patients versus CA negative (CA positive/negative patients were defined by IF staining of centrin).

Despite comprehensive data about CA in PCs, preceding B cell populations in the light of carcinogenesis are still not examined enough. Further studies of B cells with CA in different stages of their development could provide important information about MM pathogenesis.

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