Impact of Nestin Analysis in Multiple Myeloma

Význam analýzy nestinu u mnohočetného myelomu

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Summary
Nestin, a marker of multipotent precursor cells, is an important dynamic structure; its polymerization/depolymerization influences intracellular signaling and participates in key cell processes such as proliferation, migration and cell survival. It is presumed that nestin plays a central role in carcinogenesis. It is suggested that nestin might be a suitable diagnostic and prognostic indicator of malignancy and a potential marker of cancer stem cells. Unexpectedly, nestin has been identified in mature $CD138^+CD38^+$ plasma cells (PC) of multiple myeloma patients (MM). Expression of nestin, a marker of stem/progenitor cells, in malignant PC, that are considered to be terminally differentiated, indicates that nestin might play a unique role in pathology of MM.

Key words
nestin – plasma cells – multiple myeloma – myeloma stem cells – myeloma-initiating cells – flow cytometry

Souhrn
Nestin, marker multipotentních prekurzorových buněk, představuje významnou dynamickou strukturu, jejíž polymerizace/depolymerizace ovlivňuje intracelulární signalizaci a podílí se na řadě klíčových buněčných procesů, jako je proliferace, migrace a přežívání buněk. Předpokládá se, že nestin hraje centrální roli v procesu karcinogeneze. Nestin je považován za možný diagnostický a prediktivní indikátor malignity solidních nádorů a potenciální marker nádorových kmenových buněk. Překvapivě byl identifikován i ve zralých $CD138^+38^+$ plazmatických buňkách (PC) mnohočetného myelomu (MM). Exprese markeru kmenových/progenitorových buněk v maligních PC, které jsou považovány za terminálně diferencované, indikuje, že nestin by mohl hrát významnou roli v patologii MM.

Klíčová slova
nestin – plazmatické buňky – mnohočetný myelom – myelomové kmenové buňky – myelom-iniciující buňky – flowcytometrie

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Introduction

Multiple myeloma (MM) is a hematological malignancy characterized by clonal expansion of malignant plasma cells (PC) in bone marrow, production of monoclonal immunoglobulin and osteolytic lesions [1]. Despite of vast advances in myeloma treatment, the disease is still incurable [2–4]. Short median survival of patients treated with conventional therapy (4–5 years) has led to initiate a new trend in prediction and prevention of progression to malignant stage of MM [5]. Benign stage of MM is represented by asymptomatic monoclonal gammopathy of undetermined significance (MGUS) that is associated with a 1% per year risk of progression to multiple myeloma (MM) or related disease [6]. However, current MGUS risk-stratification model for accurate and early assessment of malignant transformation risk is still insufficient, as described by Klincová et al in this supplementum of Klinická onkologie. New diagnostic and prognostic parameters and detailed characterization of population responsible for the origin, progression and sustainability of the disease are required.

Myeloma progenitors/myeloma-initiating cells (MIC) are supposed to be a minor population of slightly differentiated cells reminiscent of memory B-lymphocytes with surface markers $CD19^{+}CD27^{+}CD138^{-}$ [7]. However, Yaccoby & Epstein [8] proved that also dominant population of human $CD45^{low}CD38^{high}CD138^{+}$ PC is able to proliferate and produce tumor mass. Moreover, myeloma PC show high phenotypic plasticity and are able to dedifferentiate and acquire autonomous survival properties under specific cultivation conditions [9]. The real origin of MIC is still unclear, although these data indicate that myeloma PC have ability to reprogram, reverse senescence and induce stem cell properties. This hypothesis is supported by our current results indicating that potential marker of MIC responsible for plasticity of mature PC and clinical relevant factor for MM might be nestin, a marker of stem/progenitor cells [10]. Furthermore, expression of stem/germ line cell markers, such as MAGE, KLF4, SOX2, CD117, has been already reported in monoclonal gammopathies [11–14]. Detailed characterization of MIC would enable identification of new prognostic markers for progression rate monitoring but also lead to targeted eradication of this population and delay progression to symptomatic disease. In the present work, we discuss the importance of nestin analysis for MM and describe methodological approaches for study of intracellular proteins in rare populations.

Neural Stem Cell Marker Nestin

Nestin, a class VI intermediate filament protein, was originally described as a neuronal stem cell marker during central nervous system (CNS) development [15] but currently shows a wider range of expression that has been previously thought [16–20]. Human nestin gene is located on the long arm of chromosome 1. Time- and site-specific expression of nestin is driven from promoter activated by Sp-1 transcription factor [21]. The protein has a high molecular weight (about 240 kDa), which differs among organs because of protein modifications [22]. Nestin is characterized by an α-helical central "rod" domain, typical for all intermediate filaments, short N-terminus and a very long C-terminus which could function as a linker or cross-bridge between intermediate filaments, microfilaments and microtubules [23]. Nestin is unable to self-assemble; therefore, it requires the presence of other IF proteins, such as vimentin, desmin or α-internexin [16,24,25]. Phosphorylation/dephosphorylation of nestin may modulate disassembly and assembly of intermediate filaments [26]. These processes might play a role during increased cytoplasmic trafficking in progenitor cells undergoing division or in migrating interphase cells [27–29]. Nestin was shown to participate in asymmetric redistribution of cytoskeletal proteins and other factors to daughter neuroepithelial cells [30]. Moreover, cytoprotective effect of nestin expression has been demonstrated in neuronal progenitor cells undergoing oxidative stress [31,32].

Nestin is expressed predominantly in rapidly dividing progenitor cells of embryonal and fetal tissue [17–18,33,34]. Upon differentiation, nestin becomes downregulated and is replaced by tissue-specific intermediate filament proteins but reappears transiently after injury of muscle or the CNS [34,35]. Nestin-positive cells were also found in adult tissues, such as CNS and skin where they are restricted to defined locations and may function as cellular "reserve" capable of proliferation, differentiation and migration [36,37,20]. Furthermore, increased nestin expression has been reported in various tumor cells, including CNS tumors, gastrointestinal stromal tumors, pancreatic cancer, prostate cancer, breast cancer, malignant melanoma, osteosarcomas, thyroid tumors and currently in PC of MM [10,33,38–42]. Nestin expression correlates with aggressive growth, metastasis, and poor prognosis in some tumors; it is considered to be a suitable diagnostic and prognostic indicator of malignant grade of tumors. Besides being a possible clinical marker, it has been recently shown that co-expression of nestin and CD133 might be a putative marker of cancer stem cells in neuroepithelial tumors [42–44]. Liu et al [42] first demonstrated gene expression of nestin in 5 MM patients and MM cell lines. Results referred to the existence of $CD56^{+}$ primary MM cells expressing neuronal markers, such as ne-

Tab. 1. Formula for calculating of relative levels of intracellular proteins in rare population.

QUANTIFICATION

In order to quantitative levels of fluorescence in protein+cells, relative protein expression is calculated using the following formula [78]:

Protein ratio ($MFI_{protein}/MFI_{IC}$) = MFI (protein+cells)/MFI (isotypic control)

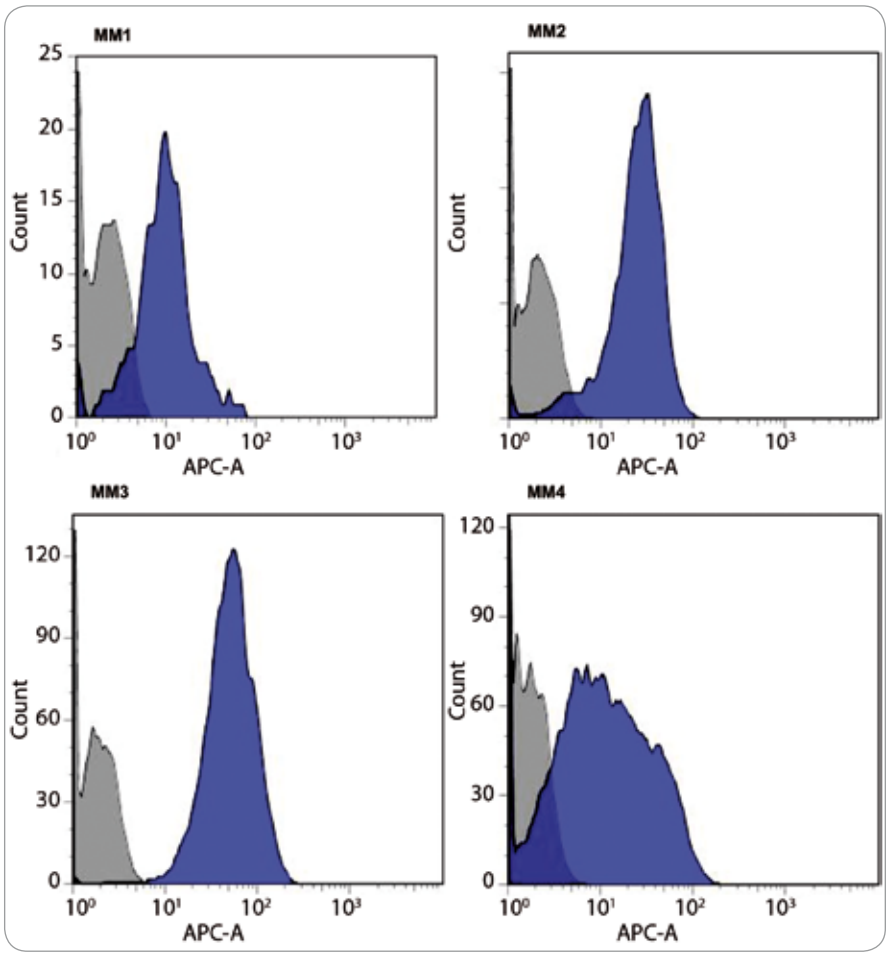

Fig. 1. Heterogeneous nestin levels in CD138+38+ PC of MM patients.
Representative results demonstrate different shifts of fluorescent intensity of entire PC population compared to isotypic control. Equivalent amounts of isotype control mouse IgG1-APC (tinted gray histograms) were assessed in parallel with anti-human nestin-APC (solid blue histograms).

stin, neuron-specific enolase and β-tubulin III. Despite of wide spectrum of nestin occurrence under normal and pathological conditions, its true biological role in cells is still unknown. Increasing importance of nestin analyses in solid tumors and missing information about expression of nestin in MM led us to evaluate nestin levels in PC *ex vivo*.

Methodology

Immunophenotypic studies were performed on bone marrow mononuclear cells (BMMNC) which were analyzed within 7 hours after sampling. BMMNC of MM patients and individuals without hematological malignancy were isolated by Histopaque® 1077-1 (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany), and stained with two-color combination of monoclonal antibodies for identification of PC – CD138-PE (clone: B-A38, EXBIO, Praha, Czech Rep.)/CD38-PE-Cy7 (Beckman Coulter, Marseille, France), based on manufacturer's instructions. After surface staining, cells were fixed with 3% paraformaldehyde (Sigma-Aldrich) for 20 min at RT, washed with PBS and followed by intracellular staining with anti-human nestin-allophycocyanin (APC) conjugated monoclonal antibody (clone: 196908, RD Systems, Minneapolis, USA) in 0.1% Triton X-100 (USB, Cleveland, OH, USA) for 1h at RT. An isotypic control IgG1-APC (RD Systems) was used each time and applied as described above for anti-human nestin antibody. Data acquisition was performed on FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) using the BD FACSDiva 4.0 software (BD Biosciences); 10^4 events/tube were collected. Nestin expression was assessed as the percentage of PC showing positive nestin staining (Nes^+PC), ratio of median fluorescence intensity (MFI_{Nes}) and median fluorescence intensity of isotypic control (MFI_{IC}) (Tab. 1).

Methodological Pitfalls in Multiple Myeloma

Proteomic analyses in MM are generally limited because of low amounts of cells. Therefore, analyses of intracellular protein levels of nestin needed to be optimized. As a suitable approach for analysis of nestin levels in PC of MM, we selected flow cytometry. This choice was made for three reasons:

1. It is a well standardized method for analyses of cell surface markers and detection of intracellular light chains (kappa, lambda) in PC [45,46].
2. It does not require high amounts of separated PC as other proteomic methods.
3. Flow cytometry has already been used for nestin detection in human gliomas [47].

In this study, the intensity of nestin expression corresponded to the mean fluorescence intensity expressed as arbitrary relative linear fluorescence channel units scaled from 0 to 10^4 of the stained cells after subtracting the mean fluorescence intensity obtained for control unstained cells. However, this method did not consider non-specific binding of nestin antibody. Van Stijn et al [48] developed an accurate analysis of intracellular proteins in combination with extracellular antigens. The method enables detection and quantification of proteins in very small cell populations using isotypic control (IgGx-fluorochrom-conjugated monoclonal antibody intended for determination of non-specific staining in flow cytometric analysis; x – IgG subclass) (Tab. 1). According this method, we showed that nestin was heterogeneously expressed among MM patients (Fig. 1). Based on percentage of nestin-positive PC and relative nestin levels, significant differences were confirmed between MM patients and the control group without hematological

malignancy but we did not find any statistically significant differences between newly diagnosed and relapsed patients based on our flow cytometry data [10].

The presence of nestin, a marker of multipotent proliferative cells, in malignant PC which are considered to be mature and terminally differentiated cells, is highly controversial. There are a few proposals for the explanation of nestin role in MM:

1. Specific stimulus of damaged microenvironment may lead to activation of "stem cell" characteristics in more matured cell than was initially supposed [49]. Induction of stem cell gene expression might support survival or give some growth advantages to the PC subset [50,9].
2. Dynamic character of nestin network plays a role in metastatic and migratory potential of solid tumors [51]. It is possible that nestin expression might be responsible for migration of PC to the extramedullary site.
3. Considering the exceptional role of PC in the immune system, nestin might have another unknown function in malignant PC that might be associated with overproduction of abnormal proteins [41].

Conclusion

Nestin may become a useful diagnostic and prognostic marker for MM; therefore, it deserves further research. Our limited information about the role of nestin in MM requires clarifying its biological implication in the pathology of MM. As our data showed, flow cytometry might be a suitable tool for analyses of relative levels of nestin and simplify proteomic analysis of intracellular proteins. Clinical application of flow cytometry is currently increasing in research of monoclonal gammopathies and has become almost routine instrument for fast and easy screening of B-cell populations in monoclonal gammopathies.

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