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Chapter 8

Bone Marrow Infiltration in Neuroblastoma: Characteristics of Infiltrating Cells and Role of the Microenvironment

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Additional information is available at the end of the chapter

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1. Introduction

Neuroblastoma (NB) is a pediatric tumor that arises from peripheral nervous system. The clinical presentation of NB is highly heterogeneous, ranging from asymptomatic tumor masses requiring little, if any, treatment to metastatic disease requiring intensive multimodal therapies (see [1] for review). Also the outcome of NB patients is highly variable. The 5-years overall survival ranges from 98-100\% for stage I infants without MYCN amplification to less than 20\% for children with stage 4 MYCN amplified tumors [2]. The main prognostic factors are indeed stage, age at diagnosis and MYCN oncogene status [3].

At diagnosis, about 50\% of cases present metastatic spread that mainly involves vascularized tissues, such as bone marrow (BM) and bone. According to the International Neuroblastoma Staging System (INSS [4]), patients with metastatic disease are categorized as stage 4, whereas in the absence of metastatic spread patients are categorized as stage 1, 2 and 3, depending on the extent of the primary tumor (within or across the midline), the involvement of ipsilateral or contralateral lymph nodes, and the surgical possibility of resection. Recently, the INSS has been replaced by the International Neuroblastoma Risk Group-Stage System (INRG-SS) based on image-defined surgical risk [5]. According to the INRG-SS criteria, patients with metastatic spread have stage M disease, while patients with localized disease have stage L1 or L2, depending on the level of surgical risk.
2. Role of bone marrow infiltration in staging of NB patients

Since the spread of tumor cells to the BM is a grim prognostic indicator for patients with NB, the search for BM infiltration is of utmost importance for both staging and therapeutic purposes. According to the INSS, presence of metastases is assessed by appropriate imaging, including $^{123}$I-MIBG scintigraphy, and morphological examination of both BM smears and trephine biopsies [4]. In spite of the limited sensitivity of morphological analysis, alternative methods for NB cell detection, such as flow cytometry, immunocytology (IC) and reverse transcription-polymerase chain reaction (RT-PCR) for markers selectively expressed in NB cells, are not included into the staging system. The reason for this choice depends on the good survival rate of children with localized NB [2], suggesting that, even if present, few circulating tumor cells are not clinically relevant. Only few patients with localized NB, in fact, relapse or die of the disease. Therefore, the introduction of more sensitive methods for the detection of BM-infiltrating NB cells may cause inappropriate overtreatment of patients with localized NB, resulting in unnecessary toxicity and long term side effects. These sensitive methods, however, are currently evaluated in ongoing clinical protocols for patients with metastatic disease for their potential prognostic value.

3. Sensitive methods for detection of neuroblastoma cells in BM samples

3.1. Flow cytometry

Total cells from BM aspirates are incubated with a monoclonal antibody (mAb) directed against a neuroblastoma specific antigen, as the disialoganglioside GD2, and with a mAb specific for the hematopoietic cells, as the pan leukocyte CD45, each one labeled with a different fluorochrome. After removal of the unbound mAbs and erythrocyte lysis, samples are analyzed in a flow cytometer. The GD2-positive CD45-negative cells, shown in Figure 1, are considered BM-infiltrating NB cells and their number relative to the total hematopoietic cells can be determined.

3.2. Immunocytochemistry (IC)

Ficoll-purified mononuclear cells from BM aspirates are spotted onto slides that are fixed and then incubated with an anti-GD2 mAb. After stain development, the stained GD2-positive cells can be counted by light microscopy, relative to a given number of total mononuclear cells. Standardized conditions for IC analysis and reporting have been developed [6]. An example of a BM slide with GD2-positive cells is shown in Figure 2.

4. Molecular analysis (qualitative and quantitative RT-PCR)

BM aspirates from the iliac crests are stored in tubes containing RNA preservative. Total RNA is then extracted and reverse transcribed (RT). For qualitative PCR analysis, the cDNA is
amplified for 35 cycles in a thermal cycler with primers specific for NB related markers, such as Tyrosine hydroxylase (TH) [7], GD2 synthase [8], PHOX2B [9], and for a housekeeping gene (GAPDH, β2-microglobulin). An aliquot of each PCR reaction is then loaded onto a 2% agarose gel and electrophoresed. The amplification products are visualized by staining with ethidium bromide. A sample is considered positive for the tested gene if an amplification product of the expected size is present, a sample is considered negative if product for the tested gene is absent and the product for the housekeeping gene is present, as shown in Figure 3.

Figure 1. Cytofluorimetric analysis of BM cells from a patient with stage 4 NB, showing the presence of GD2-CD45+ normal hematopoietic cells (upper left panel 74% of total cells) and GD2+CD45- NB metastatic cells (right bottom panel 26% of total cells).
Figure 2. Immunocytological analysis of a BM smear from a patient with stage 4 NB, showing the presence of rosettes of NB cells stained in red and normal unstained hematopoietic cells (Magnification 20X).

Figure 3. Representative agarose gel electrophoresis of PCR products. Samples 1, 2 and 7 are positive for TH expression, whereas samples 3, 4, 5 and 6 are negative. M: molecular weight DNA markers.

For quantitative analysis (qPCR), the cDNA is amplified for 40 cycles in triplicates in a real-time thermal cycler, using primers specific for a NB related marker and for a housekeeping
gene, in the presence of specific probes labeled with different fluorochromes. If a cDNA specific for the NB marker is present, at each amplification cycle the probe-specific fluorescence is released and detected by the instrument. At the end of 40 cycles, the cycle at whom the released fluorescence overcomes the threshold is used to quantitate, through an algorithm [10], the relative amount of the NB-specific amplified product. An example of quantitative analysis is shown in Figure 4. Standardized conditions for RT-qPCR analysis and reporting have been developed [11].

Figure 4. Representative amplification plots showing fluorescence amounts in relation to the number of cycles. Curves of the same color correspond to the replicates of the same sample. On the right side of the figure the plate layout is displayed.

4.1. Sensitivity, specificity, diagnostic accuracy and prognostic values of different detection methods

The sensitivity of morphological analysis is approximately 1 NB cell out of $10^3$-$10^4$ for smear and bone trephine biopsy, respectively [12]. Sensitivity of flow cytometry, immunocytochemistry and molecular analysis is evaluated in spiking experiments by mixing logarithmic dilutions of a NB cell line with fixed amount of mononuclear cells from a healthy donor. Sensitivity of flow cytometry is 1 out of $10^4$ cells [13], whereas that of both IC and RT-qPCR is 1 out of $10^6$ cells [11, 12].
Specificity of flow cytometry and immunocytochemistry is assessed by negative results obtained in samples from healthy donors. For molecular analysis specificity is assessed by negative results in samples reverse transcribed without reverse transcriptase and in samples amplified without cDNAs.

4.2. Comparison of different techniques and markers

The sensitivity and specificity of different techniques and of different markers have been compared [12, 14-19]. Following the development of standardized conditions [6], GD2 is the marker of choice for IC analysis [20]. GD2-IC is currently evaluated for its prognostic significance in ongoing protocols for stage 4 NB patients, both in Europe and North America.

To overcome NB tumor heterogeneity and increase sensitivity and specificity of RT-PCR analysis, different panels of various NB-specific markers have been developed thanks to the microarray technology [9, 21, 22]. To date the panels have not been compared, but each panel is currently tested for its prognostic role in ongoing therapeutic protocols for stage 4 NB patients.

5. BM infiltration in patients with localized NB detected by sensitive methods

Patients with localized NB without MYCN amplification have good overall survival (OS) rates, following either surgery alone (stage 1 and 2, 95% and 86% OS, respectively), or standard-dose chemotherapy followed by surgery (stage 3, 65% OS) [23]. Both the histological features of the tumors [24] and the presence of no random genetic abnormalities [25] in the primary tumor are highly prognostic in patients with localized NB. Thus, these two parameters are presently evaluated at diagnosis to stratify the patients with localized disease into different therapeutic regimens. Some of the patients with favorable histology and genetics, however, relapse, making the search for new prognostic markers still necessary [3, 20].

Since conventional morphological methods have limited sensitivity, it has been suggested that some of the patients with localized NB could have a low number of metastatic cells that could be responsible for relapse. If such hypothesis was true, the use of sensitive methods, such as IC and RT-qPCR, may be helpful in identifying patients at risk of relapse and death. Indeed, we observed that in patients with localized NB the presence of GD2 positive cells in BM samples at diagnosis negatively associated with survival [12]. Since this finding was based on a small sample size with a relatively short follow-up, a further study performed in a larger cohort of patients confirmed the negative impact of BM GD2-positive cell infiltration on survival of patients with localized NB [26]. Moreover, the negative impact was demonstrated to be independent of MYCN amplification (Figure 5), the most important negative prognostic factor for these patients [3]. It is worth noting that MYCN amplification is a relatively rare event, occurring in about 10% of patients with localized NB [24, 27-29], making it inadequate to identify all the patients who will eventually relapse.
Peripheral blood (PB) samples can be obtained without patient’s sedation with evident advantages as compared to BM samples. Thus, we tested whether RT-qPCR analysis for NB-related markers in BM and PB samples from patients with localized NB had a prognostic impact on their survival [30]. The expression of seven different genes, previously shown to be specific for NB cells [7, 9, 13, 14, 18, 31-35], was evaluated and compared to those of healthy subjects. A high percentage of samples resulted positive for the various NB-related markers (Figure 6). Since the patients’ cohort had a fairly good survival rate, in accordance with literature data [3, 36, 37], the positive results were likely related to low transcription levels by the PB hematopoietic cells [21, 22, 32, 38], or were due to the existence of so-called dormant cells, i.e., tumor cells unable to proliferate [39].
Figure 6. Expression of various NB-related markers in BM and PB samples from patients with localized disease and healthy children.
In the attempt to discriminate between illegitimate transcription by hematopoietic cells and transcription by low numbers of NB cells, ROC analysis was applied to find cut-off levels able to discriminate patients with localized NB from healthy subjects. Although the use of cut-off levels for each NB-related marker in PB and BM samples increased their specificity, the percentage of positive results that did not correlate with clinical events remained high. Also a modified ROC analysis [40] failed to improve the prognostic value of RT-qPCR analysis for any of the tested marker. However, TH expression in PB samples significantly correlated with worse EFS of patients with localized NB (Figure 7).

Figure 7. Event-free survival of patients with localized disease stratified by TH status in peripheral blood samples.
Indeed, \( TH \) expression was significantly higher in relapsing patients than in patients that remained in complete remission. Similar results were reported by Yanez et al. [41] that tested BM and PB samples from patients with localized NB for \( TH \) and \( DCX \) mRNA expression. Although the use of multiple markers has been recommended [20], the results obtained in these studies indicated that, in the subset of patients with localized NB, molecular analysis should be limited to \( TH \) expression in PB samples, because multiple target analysis did not add useful information to that obtained by \( TH \) analysis alone.

In conclusion, in patients with localized NB, detection of metastatic cells in BM by means of GD2-IC analysis and detection of \( TH \) mRNA expression in PB samples significantly associated with a higher risk of relapse. Therefore, both analyses may help individuating patients at risk of relapse that may require a closer follow-up.

6. BM infiltration in patients with metastatic disease

Presence of metastasis at diagnosis in children over 18 months of age is a powerful prognostic factor for a poor outcome [2, 3]. In order to understand the mechanisms responsible for such aggressive behavior, an extensive characterization of primary tumor cells from stage 4 NB patients has been performed. DNA abnormalities [42, 43], gene expression profiles [44-46], and non-coding RNAs expression [47, 48] have been proposed as sensitive indicators of NB tumor aggressiveness. Unfortunately, all the gene classifiers, although validated on independent patient cohorts [46, 49], did not appear to be helpful in stratifying stage 4 patients into different risk groups. Moreover, whole genome sequencing of primary NB tumors [50] demonstrated that no specific mutations or chromosomal alterations were present in NB tumors, suggesting that the mechanisms responsible for invasiveness and metastasis should be searched elsewhere.

6.1. Role of the BM microenvironment for NB metastatic invasion

The processes of invasion, survival and proliferation at distant sites may be mediated in part by the microenvironment. In the BM, several cell types, such as adipocytes, stromal and endothelial cells are present together with cells of all hematopoietic lineages. Each cell type may secrete factors that affect several signaling pathways leading to modifications in BM structural organization and cell function. Among these factors, the CXCL12 chemokine has been proposed to play an important role in NB invasiveness [51-53], but conflicting results have been obtained [54, 55]. Thus, we decided to compare the gene expression profiles of resident BM cells from healthy children to those of BM cells from patients with localized and metastatic NB. The results indicated that the resident BM cells from patients with either localized or metastatic NB have a different genetic signature from healthy children. However, the deregulation of transcription was more evident in the BM microenvironment of patients with metastatic stage 4 disease (Figure 8) [56].
Figure 8. Hierarchically clustered heat maps of differentially expressed genes in BM cells from stage 4 patients (infiltrated BMs, I), normal BMs (N) from healthy children, and BM cells from localized NB patients (non-infiltrated BMs, NI). Each color patch represents the expression level of genes (row) in that sample (column), with a continuum of expression levels from bright green (lowest) to bright red (highest).

Precisely, BM samples from NB patients significantly overexpressed genes involved in the innate immune responses. In particular, all NB patients expressed an interferon (IFN) signature [57]. IFNs are pleiotropic cytokines involved in different biological processes [58], and IFN signatures have been associated with tumor progression of melanoma and colorectal cancer [59]. Moreover IFN signatures associated with the worse prognosis of African-American patients with prostate [60] and breast [61] cancer as compared to Caucasian patients. The IFN signature includes genes involved in the defense against bacterial and viral pathogens [62, 63]. Since all NB patients showed the IFN signature it was proposed that the NB primary tumor may release, or induce the release of, soluble factors as occurring during an infection. The BM microenvironment of NB patients up-regulated also the IFN related DNA damage resistance signature (IRDS), that was shown to be associated with resistance to radiation-induced DNA damage [64], as summarized in Table 1.
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Table 1. List of the most relevant genes of the IFN and IRDS signatures overexpressed by resident BM cells from patients with metastatic (I_BM) and localized (NI_BM) neuroblastoma.
In conclusion, children with NB have evidence of chronic inflammation, more intense in the presence of infiltrating NB cells [56], that may reduce anti-tumor immune responses and promote tumor progression [65].

Furthermore, resident BM cells from patients with NB down-regulated genes involved in cell adhesion, in erythrocyte, myeloid and platelet differentiation, and most importantly, CXCL12 expression. The CXCL12 down-regulation reached near complete silencing in patients with metastatic disease (Figure 9), likely explaining the anemia and platelet dysfunctions observed in stage 4 patients.

Figure 9. Expression of CXCL12 in BM samples from healthy children (H_BM), and children with localized (NI_BM) and metastatic (I_BM) neuroblastoma.

The CXCL12 mRNA down-regulation was independent of direct contact between neuroblasts and resident BM cells, as expected from the down-regulation observed in patients with localized NB. Since it is known that CXCL12 expression is regulated by the circadian secretion of noradrenaline [66], we speculated that CXCL12 down-regulation may be dependent on noradrenaline secretion by NB tumor cells [67]. Although, the CXCL12 chemokine has been proposed to play a pivotal role in promoting the homing of the CXCR4 positive NB cells in the
BM [51, 68], the absence of CXCL12 in the BM of NB patients makes highly unlikely that the axis CXCL12/CXCR4 play a role in the BM infiltration by NB cells. It is worth noting that we had previously shown that CXCR4 in freshly isolated human metastatic NB cells was not functional [54], and that Carlisle et al. [55] showed that CXCR4 expression was regulated independently of CXCL12.

In conclusion, NB tumor growth at the primary site can alter the BM microenvironment, and the presence of BM-infiltrating NB cells makes these alterations only more pronounced. Therefore, the BM microenvironment is unlikely to be responsible for the presence of NB cells in the BM.

6.2. Characteristics of the BM-infiltrating NB cells

After in vitro culture of BM samples from patients with metastatic disease, Hansford et al. [69] isolated cells endowed with high tumorigenic potential, suggesting that metastatic cells may be enriched in tumor initiating cells (TICs). A gene expression profiling of TICs has been reported [70]. However, it has been demonstrated that the isolated TICs were not NB cells [71], and Coulon et al. [72] had recently demonstrated that in vitro expanded stem-like NB cells were a dynamic and heterogeneous cell population, quite difficult to characterize because of the influence of external stimuli.

To avoid any modification or selection following in vitro culture, we thus decided to characterize freshly isolated BM-infiltrating NB cells. The metastatic cells expressed several co-stimulatory molecules [73] and were susceptible to NK cell-mediated lysis [74]. Moreover, as mentioned above, the CXCR4 expressed by these cells was not functional [54].

Since the proteins selectively over-expressed by the BM-infiltrating NB cells may represent novel prognostic markers and potential targets for biologically driven therapy for metastatic NB patients, we performed gene expression profiling of these cells, as compared to the cells in the primary tumors [75]. The results of the study showed that the BM-infiltrating GD2-positive cells were enriched in CD56-positive and NB84-positive mononuclear NB cells, had the same genetic aberration as the primary tumor cells, and expressed NB-specific genes as primary tumor cells. The BM-infiltrating GD2-positive cells up-regulated several genes normally expressed by different lineages of resident BM cells. Therefore, to ascribe the expression of the proteins encoded by these genes to the metastatic NB cells, we took advantage of multiple color cytofluorimetric analysis of unprocessed BM samples from stage 4 patients. While in unprocessed BM samples from healthy individuals the GD2 expression was absent [76], in BM samples from stage 4 NB patients the GD2-positive cells represented about 20-30% of mononuclear cells. These latter cells never express the pan-leukocyte CD45 antigen and always co-express the NB specific markers B7H3 and CD56 [77, 78] (Figure 10), thus confirming that the GD2-positive BM-infiltrating cells were indeed metastatic NB cells. All freshly isolated GD2-positive BM-infiltrating NB cells never expressed CD133, sometime co-expressed c-KIT, CD37 and CD177, but most importantly, they always expressed both HLA-G and calprotectin, as shown in Figure 10.
Figure 10. A representative two color cytofluorimetric analysis of a fresh BM sample from a patient with metastatic stage 4 NB, tested with anti-GD2 mAb and: anti-B7H3 (5B14), anti CD45, anti-CD56, anti-CD117 (c-kit), anti-CD37, anti-CD177, anti-HLA-G, and anti-calprotectin. Each plot shows specific mAb fluorescence intensity (Y axes) versus side-scatter (X axes), after gating on GD2 positive cells.
The heterodimer protein calprotectin, encoded by the \textit{S100A8} and \textit{S100A9} genes, is a member of the S100 family, composed of small (10–12 kDa) acidic calcium and zinc binding proteins. Calprotectin is normally expressed by phagocytes and polymorph nuclear leukocyte and it is released into biological fluids during inflammation. Calprotectin, in fact, is widely used as a biomarker of inflammation [79]. Calprotectin is a potent ligand of the Toll-like receptor 4 (TLR4) [80], which is responsible for specific response to endogenous danger signals. Thus, the expression of calprotectin by the BM-infiltrating metastatic cells may be responsible in part for the state of chronic inflammation of the BM microenvironment (see previous paragraph for details). Moreover, the calprotectin-TLR4 axis may also guide metastatic cell invasion, and facilitate the survival and proliferation of cancer cells at the metastatic site [79].

The GD2-positive BM-infiltrating cells also expressed HLA-G, a monomorphic HLA class Ib molecule, whose over-expression facilitate tumor escape from host immune response in a wide variety of human cancers [81]. Since primary NB tumors did not express HLA-G [82], it is conceivable that HLA-G may contribute to the high aggressiveness of BM-infiltrating NB cells throughout its immunosuppressive activity.

\section*{7. Conclusion}

Presence of NB cells within the BM is the most powerful negative prognostic factor for patients with NB. Presently, sensitive methods of detection of metastatic cells have been standardized [20], and prospective studies are ongoing to demonstrate their relative or combined prognostic role. In the near future these methods will help stratification of stage 4 patients into different risk groups. Conversely, these sensitive methods are of limited use in patients with localized NB.

To date, information about the role of the BM microenvironment in driving infiltration by metastatic cells is few and still conflicting. However, the finding that the BM microenvironment of patients with localized disease is not so different from that of patients with metastatic disease [56], strongly support the hypothesis that the invasion of the BM mainly depends on the characteristics of the metastatic cells, rather than on the properties of the BM microenvironment. In this regard the findings that the BM-infiltrating NB cells expressed proteins not found in the primary tumor cells is intriguing [75]. In fact, both calprotectin and HLA-G favor tumor escape from anti-tumor immune responses, likely contributing to the survival and proliferation of the metastatic cells in the BM. These proteins may be also responsible for the state of intense chronic inflammation observed in patients with metastatic NB [56]. Future studies are needed to elucidate the mechanisms responsible for the acquisition of the different properties of metastatic cells as compared to primary tumor cells. However, the proteins specifically expressed by BM-infiltrating metastatic NB cells could be new prognostic markers and novel therapeutic targets for high risk NB patients.
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References


De Bernardi, B, Mosseri, V, Rubie, H, Castel, V, Foot, A, Ladenstein, R, et al. Treatment of localised resectable neuroblastoma. Results of the LNESG1 study by the


