Epstein-Barr virus encoded BARF1 protein: immunopathogenic role, transcriptional regulation and diagnostic use in nasopharyngeal carcinoma

Hoebe, E.K.

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Discussion and future perspectives

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In carcinomas associated with EBV the virus has a highly restricted gene expression pattern. A specific transcript observed in a majority of carcinomas, but not in lymphomas is derived from the BARF1 gene. Although the encoded BARF1 protein has been described as a viral oncogene, the role of this protein in oncogenesis is not completely unraveled yet. The natural BARF1 protein was recently defined as a complex secreted hexameric structure with post-translational modifications in the form of glycosylation and a deleted N-terminus (here called sBARF1) (1). Previous studies used recombinant BARF1 protein produced in diverse expression systems like *E.coli*, yeast or *Spodoptera frugiperda* insect cells, and was often tagged which could disturb processing and folding. The research in this thesis was largely performed with sBARF1 protein expressed in human epithelial cells and based on the sequence as found in patients with NPC (2).

BARF1 in EBV associated tumors was previously described to have an immune modulating role predicted by the partial structural homology with growth factor receptors (3). Our research confirmed that sBARF1 functions as an immune modulator by acting as decoy receptor for macrophage colony stimulating factor (M-CSF), functionally inhibiting cells of the myeloid lineage. Mutations in regions of BARF1 predicted to interact with M-CSF disturbed sBARF1 function. The detailed interaction domains of sBARF1 and M-CSF were published in parallel (4,5). Our newly developed antibodies to sBARF1 were able to functionally block the M-CSF interaction with sBARF1 reducing its immune modulational function. Since NPC is characterized by a high number of macrophages in the tumor field (6,7), activation of these macrophages and other myeloid cells by blocking of sBARF1 activity might result in immune activation of the currently inactive T lymphocytes in the tumor environment. Therefore inhibition of the BARF1 M-CSF interaction could be a potential therapeutic target. Future studies should investigate whether BARF1 levels indeed correspond to the immunological picture and possibly to disease outcome in patients with NPC and GC.

The role of BARF1 in the EBV life cycle and specifically in EBV associated carcinomas is reflected in its cell type specific transcription activity. We analyzed why BARF1 is only transcribed during latency in EBV associated carcinomas and not in any of the EBV associated lymphoid malignancies. During latency, EBV silences and regulates gene transcription by promotor methylation. We demonstrated that the BARF1 promotor is highly methylated, independent of its cellular background and expression level. Evaluation of the BARF1 promotor area for binding sites of cell type specific transcription factors directed us to the p53 family of transcription factors. Our results showed that the epithelial differentiation marker ΔNp63α, and none of the other p53 family members, was capable of BARF1 transactivation. ΔNp63α is highly expressed in undifferentiated NPC tumors (8-10), and multiple EBV genes have been described to influence differentiation via β-catenin and ΔNp63α (11-16). The undifferentiated phenotype of the cell is vital to maintain EBV in a latent state. A differentiation-sensitive response element is present in the promotor of the

immediate early gene BZLF1 (17,18). Differentiation and subsequent growth arrest induce the viral lytic cycle during which all EBV genes are expressed. These highly immunogenic lytic viral proteins trigger an immune response, limiting viral persistence. Therefore differentiation of the host cell is tightly controlled by multiple EBV genes by regulation of ΔNp63α and β-catenin (11-16). ΔNp63 is thus a continuously active factor in EBV infected epithelial cells and showed in our study to increase transcription of BARF1. BARF1 was previously shown to stimulate expression of NF-kB and cyclin D1, both providing pro-oncogenic stimuli and promoting EBV latent persistence and cell survival (19,20). However, ΔNp63α alone was not able to induce BARF1 transcription in the context of the viral genome indicating that a cooperation with other transcription factors is necessary. To elucidate the identity and importance of unknown host or viral factors in BARF1 gene regulation, we propose to perform DNA pulldown experiments using the BARF1 promotor area combined with mass-spec proteomic analysis. Future studies might address whether epigenetic control by the level of promoter methylation and histone modifications are involved in BARF1 regulation (21,22).

In contrast to the complex cell type specific regulation of BARF1 during viral latency in epithelial cells, we showed that BARF1 transcription is directly activated by the immediate early BRLF1 protein (R) during lytic replication. BARF1 is expressed in the early phase, indicating that BARF1 has functional relevance early on during lytic replication which might relate to its immune modulating role. Inhibition of M-CSF by BARF1 during replication might not only hamper clearance of the virus by the immune system, it might even allow monocytes or macrophages to act as virus transmission vehicle of internalized or bound virus (23,24). This possibility should be considered in future experiments. Recent studies by Ohashi et al using the EBV-related lymphocryptovirus (LCV) in rhesus macaques indicate that BARF1 is also an important factor during primary infection (25). Functional BARF1-inhibitors or BARF1-blocking antibodies might reveal whether it is the immune modulating decoy-function of BARF1 that is responsible for this.

Since the BARF1 protein most likely is secreted by EBV positive carcinoma *in vivo*, BARF1 is potentially a relevant diagnostic marker for early detection of NPC and GC. Detection of the secreted BARF1 protein, would have a major impact on NPC screening and early diagnosis, and the presence of anti-BARF1 humoral immune responses might supplement current NPC serology (26-28). Given that NPC is located in the nasal-oral cavity, secreted protein from the tumor might possibly be detected in saliva, opening options for the development of a noninvasive dipstick test. Purification of native sequence sBARF1 protein secreted from a human expression system has enabled us to define whether NPC patients have antibodies to BARF1. Our results show that, although there is a significant difference between antibody responses of NPC patients compared to healthy volunteers, the low immunogenicity of the BARF1 protein makes this serology approach unsuitable as a diagnostic assay for early detection of NPC. For detection of the secreted protein specific antibodies capable of detecting non-denatured native BARF1 were developed and used to set up an antigen-capture ELISA system. This assay revealed positivity in a very limited number of NPC patients,

despite high levels of BARF1 RNA present in these tumors. The capture ELISA in its current form can detect sBARF1 levels above 10 ng/ml, which appeared not sensitive enough to conclusively state that sBARF1 is present in NPC patient sera at levels above those found in healthy EBV carriers. One earlier rapport suggests that sBARF1 is present in saliva of NPC patients up to 5000 ng/ml, but the methods and antibodies used are highly controversial (29). The MS/MS proteomics approach proved even less sensitive with a detection limit of 1000 ng/ml, but revealed a peptide that can be used to set up a targeted quantitative approach in the future. Detection of sBARF1 in patient material has proven to be a challenge. The question whether the BARF1 protein is transcribed *in vivo* was not fully answered in this thesis. However, the presence of antibodies to BARF1 in NPC patients is indirect evidence that the protein is present. It is very well possible that antibody based detection of BARF1 is hampered by binding partners shielding epitopes or because it is rapidly degraded by opsonization.

In this thesis we showed that sBARF1 is a decoy receptor for M-CSF and has an immune modulating function. The transcriptional regulation as an early gene by R and cell type specific by ΔNp63 indicate that BARF1 has functional relevance for EBV during lytic replication as well as during latency in epithelial cells. The presence of antibodies to BARF1 in NPC patients is indirect proof that the BARF1 mRNA present in tumor is transcribed into protein *in vivo*, but the protein itself remain elusive, despite the use of new polyclonal and monoclonal antibodies capable of recognizing and capturing hexameric native sBARF1 protein.

Future perspectives

Our studies on BARF1 have shown that secreted sBARF1 functionally inhibits M-CSF induced differentiation of mononuclear cells *in vitro*. To make a case for a BARF1 targeted therapy, the relative contribution of sBARF1 to the immunosuppressive environment in the tumor should be investigated. It will be important to establish whether BARF1 levels indeed correspond to the immunological picture of local tumor immune evasion and possibly to disease outcome in NPC and GC patients. A hurdle in this detection of BARF1 protein is the necessity to use antibodies. BARF1 RNA in situ hybridization might be an option worthwhile exploring. A NPC humanized mouse model might also give us more insight in the net contribution of sBARF1 to the immunological state of the tumor. While sBARF1 is capable of capturing mouse M-CSF, it does not functionally inhibit it (4), thus M-CSF humanized mice should be used (30). As BARF1 has structural homology with other growth factor receptors it is worthwhile to further characterize sBARF1 binding partners. Other factors BARF1 functionally inhibits might elucidate why BARF1 is an important gene in EBV carcinogenesis. Pulldown experiments combined with mass spec proteomics might reveal these factors. Inhibition of the BARF1/M-CSF interaction might be a suitable immunotherapeutic strategy for NPC and future studies should involve molecular design to develop an inhibitor for this interaction. This inhibitor should also be evaluated for its effect on primary infection. On the

other hand might sBARF1 itself, as a highly stable and low immunogenic protein, be considered as a potential biotherapeutic protein to treat M-CSF related inflammatory disorders, such as rheumatoid arthritis (31-33).

Various studies describe growth promoting and anti-apoptotic properties of BARF1. So far there is no consensus mechanism of action of these properties, and the domains of BARF1 described to be involved vary between the N-terminal part remaining intracellular after cleavage and secreted BARF1 in cis or in trans (34). To further define sBARF1 as suggested mitogenic growth factor would require identification of a putative BARF1 receptor as well as a signaling pathway underlying this mitogenic effect. Future research should consider the intracellular N-terminal part to play a role in the putative proto-oncogenic effects of BARF1.

Despite our excellent antibodies to hexameric native sBARF1, we could not show sBARF1 protein in sera and tumor material using immunological methods, possibly because the epitopes are shielded from detection by complexing with host proteins including M-CSF. Future studies aiming at BARF1 detection using antibodies should further investigate if BARF1 protein is indeed blocked by serum proteins. Focus for detection of BARF1 in NPC serum or plasma should use a targeted proteomics approach, using the main identifier peptide described in this thesis.

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