האוניברסיטה העברית בירושלים
The Hebrew University of Jerusalem

Fondation de bourse de recherche sur la sclérose amyotrophique latérale (maladie de charcot) à la mémoire de Jacques Kluger

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Research report as submitted by: submitted by Professor Yosef Gruenbaum, PhD, Department of Genetics, The Hebrew University of Jerusalem

The molecular basis of the laminopathic-based Charcot-Marie- Tooth disorder – from a mutation to a potential treatment

Introduction

The Charcot-Marie-Tooth (CMT) disorders, also known as "hereditary motor and sensory neuropathies" (HMSNs) form the most common group of inherited neuropathies, affecting 10–40/100,000 individuals. The CMT disorders can be divided into two subtypes: demyelinating (i.e., type 1, or CMT1) and axonal (i.e., type 2, or CMT2) neuropathies. Genetically, CMT2 is a heterogeneous group of peripheral neuropathies [1]. Seven genes and thirty loci have been reported for CMT1. In contrast, only two specific genes responsible for autosomal dominant CMT2 (ADCMT2) and one gene for authosomal recessive CMT2 (AR-CMT2) have been identified. These include mutations in the type IV intermediate filaments (IF) gene *neurofilament-light* (*NF-L*) gene, (MIM 162280), microtubule kinesin superfamily member motor protein *KIF1B*b (MIM 118210), and the type V nuclear intermediate filament gene *LMNA* (MIM 150330), which encodes lamins A/C. Besides these mutations, gene defects in *MPZ* (MIM 118200), which encodes myelin protein zero, causes both CMT1 and CMT2 phenotypes.

The mutations in the *LMNA* gene cause AR-CMT2, which is a rare and severe condition. Clinically, the main symptoms in 90% of cases are early onset of the disease, symmetrical muscle weakness and wasting (predominantly in the distal lower limbs), foot deformities, and walking difficulties associated with reduced or absent tendon reflexes. Confirmation of diagnosis relies essentially on electrophysiology, which shows NCVs 138 m/s at the median nerve, and on histopathology after nerve biopsy, which evidences a loss of myelinated fibers with (for AD-CMT2) or without (for AR-CMT2) regenerative attempts. The amino acid substitution R298C in *LMNA* encoding lamins A/C causes AD-CMT2 [2]. Mutations in other residues in the LMNA gene cause 11 other diseases, collectively termed laminopathies, including AR-Emery Dreifuss and limbgirdle

muscular dystrophies, heart-hand syndrome, dialeted cardiomyopathy, partial 2 lipodystrophy, Seip syndrome, diabetes, Mandibuloacral Dysplasia, Hutchison-Gilford and atypical Werner progeria syndromes and Restrictive Dermopathy [3, 4].

It is still unknown why mutations in specific residues in the lamin A isoforms cause specific diseases and what are the relationship between a specific mutation and the phenotypes that it cuses. The structural model suggests that changes in lamin filament assembly causes weakening in the scaffold of nuclei leading to cell death. The gene expression model suggests that specific transcriptional regulators interact with lamins A/C. For example, a specific mutation in lamins A/C causes a loss or abnormal activity of a specific transcriptional regulator and cell death of specific cells. The cell proliferation model suggests that lamins A/C mutations affect the regulation of the cell cycle in specific type of cells [5].

Mammals have a highly complex nuclear lamina system comprising of 3 lamin genes and probably hundreds of lamin-interacting proteins, which makes the understanding of the molecular basis of CMT2 extremely difficult. In contrast, *C. elegans* have a simple evolutionarily conserved nuclear lamina [6, 7]. In addition, genetic analysis in *C. elegans* is relatively simple. Thus, studies of lamin in *C. elegans* can address fundamental questions regarding: why mutations in lamins cause death of nerve cells, which transcriptional regulators in the nerve cell require R298 for its interaction, do nerve cell nuclei change their shape due to CMT2 mutations and how lamin assembly into filaments is affected by the R298C mutation. The combined research in mammalian cells and in *C. elegans* should provide a better understanding of the disease and will suggest ways for treating it.

Another complication to the story of laminopathic diseases is the fact that mutations in lamin cause abnormal post-translational modifications of its carboxyl terminus, leaving a fraction of arnesylated molecules (reviewed in [4].

Experiments performed during the past year

The 3-D supramolecular organization of lamin filaments *ex vivo*.

We have further refined the structure of lamin both *in vitro* and *in vivo*, by testing the effects of mutations on lamin filament assembly. In previous years we have employed cryo-electron tomography (cryo-ET together with Dr. Medalia now at the University of Zurich) and determined the supramolecular structure of the diseasemutant lamin filaments assembled in *Xenopus* oocytes [8]. In the previous year, for the first time, we have started studying the structure of the lamin *in vivo* in somatic cells. Initial data shows that it is possible to get thick frozen sections of adult C. elegans for cryo-ET.3

Interactions between the lamin and BAF

BAF binds lamin and LEM-domain proteins. It is a conserved 10-kDa chromatin associated protein essential for cell proliferation. It was first identified as an essential component for retroviral DNA integration. The BAF homodimer has two sites for *in vitro* binding double-stranded DNA that can condense long DNA by "looping" it. BAF also binds histone H3, histone H1.1, lamin A and transcription regulators, as well as emerin and other LEM-domain nuclear proteins. BAF influences higher order chromatin structure and represses transcription at specific promoters. Over expression of BAF caused histones to be underacetylated and hypermethylated suggesting that BAF is an epigenetic regulator. Endogenous human and *Xenopus* BAF are post-translationally modified by phosphorylation and they become hyperphosphorylated during mitosis. This phosphorylation inhibits BAF binding to emerin.

This modification also reduces BAF binding to the lamin A tail *in vitro*, suggesting that it weakens BAF-emerin–lamin interactions *in vivo*. Dephsphorylation of BAF occurs in two mechanisms: inhibiting VRK-1 with LEM-4L and direct dephosporylation of BAF by interaction of LEM-4L and PP2A with BAF. Finally, BAF-1 is involved in the DNA damage response. However, little is known about how the binding of BAF-1 to different proteins affects its dynamics and localization. An earlier study [9] identified specific conserved BAF residues as modulators of binding affinity to lamin, various histones, DNA and emerin. To determine how mutations in specific conserved residues in BAF-1 can affect BAF-1 localization and dynamics in *C. elegance*, we have generated strains mutated in these key BAF-1 positions. These mutant G25E and L46E BAF-1 (residues which important for BAF binding to DNA and emerin) caused BAF-1 to become more nucleoplasmic. In response to heat shock these mutations caused BAF-1 to become immobile and aggregated. In order to study if these specific conserved residues can affect the interactions between BAF-emerin and BAF-lamin *in vitro*, we used BIAcore method. We showed 4 that BAF-1 S4E had a much lower affinity to lamin, indicating that phosphorylation at residue S4 in BAF-1 can decrease its ability to bind lamin but not to emerin.

Proposed experiments

For the next year we propose to:

1. Continue to study the effects of laminopathic mutations on gene expression and positioning *in vivo*.
2. Study the effects of laminopathic mutations on cellular metabolism.

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La Fondation de bourse de recherche sur le cancer à la mémoire de Modko Rozenbaum

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Research report as submitted by: Eli Pikarsky, MD PhD, Department of Immunology and Cancer Research, Institute for Medical Research Israel-Canada, The Hebrew University of Jerusalem

Introduction: Liver cancer is one of the leading causes of cancer death and is particularly resistant to treatment. Previous findings in our laboratory identified a specific signaling pathway (called NFkappaB) activation of which seemed to play a key role in the pathogenesis of this terrible disease (Pikarsky et al Nature, 2004). This is supported by mutations in several human tumors which should cause activation of the pathway. Surprisingly, in other mouse models of liver cancer, the same pathway seems to inhibit tumorigenesis. This greatly diminishes the ability to use drug that modulate NFkappaB for therapy. Our aim in this project is to understand how come this protein, which is usually oncogenic, is playing a tumor suppressive role. Our findings, detailed in the attached report show a new role for the NFkappaB pathway: improving the ability of the cell to repair damaged DNA. Thus, it can help cells overcome massive DNA damage which is incurred by carcinogen treatment. This is of particular importance as many of the chemotherapeutic agents which are used today in the clinic act by inducing DNA damage. Our findings provide 2 key concepts regarding modulation of the NFkappaB pathway:

1. Is should be worthwhile and safe in liver cancer, once exposure to carcinogens is eliminated;
2. It should be considered as an adjunct to chemotherapeutic agents which induce DNA damage.

Background: We have shown that inhibiting NF-κB in vivo blocks tumorigenesis in mouse models of cancer. Several recent clinical studies have identified recurrent mutations which result in constitutive NF-κB activation, particularly in multiple myeloma and glioblastoma. However, it is now evident that in some cases, particularly in mouse liver and skin cancers, NF-κB blockade paradoxically facilitates carcinogenesis. These opposing actions of NF-κB, even in a particular organ system, hinder the implementation of NF-κB inhibition in human studies. It is therefore important to decipher the pathogenetic mechanisms that underlie these opposing outcomes. To this end, we are using different animal models of cancer, in which we

can inhibit NF-κB or else constitutively activate NF-κB signaling in a timed and reversible manner. We found that NF-κB exerts its opposing effects at different time points in the malignant process, possibly through activation of different cellular responses.

Specific aims: We aim at the identification of the molecular processes that underlie the anti-tumorigenic properties of NF-κB.

Progress during the report period: In our current studies we compared two different models of mouse liver carcinogenesis, exposure to the carcinogen diethylnitrosoamine (DEN) and Mdr2-deficiency-based chronic liver inflammation. Using a switchable NF-κB inhibiting transgene, our results suggest that the role of NF-κB in liver tumorigenesis is time and context dependent and that NF-κB plays a role in DNA damage derived cell cycle arrest. Our results show that NF-κB's role in tomurigenesis is diverse and is dependent upon the environmental stress that initiates or promotes tumor growth. In the Mdr2-/- model, transgenic inhibition of NF-κB attenuates but does not completely eliminate tumor progression. Thus, we could study tumors that did develop in spite of alleged NF-κB inhibition. In half of the tumors arising in NF-κB inhibited Mdr2-/- mice the expression of the NF-κB inhibiting transgene is eliminated. By contrast, suppression of transgene expression rarely occurs in tumors that develop due to carcinogen exposure. This indicates that NF-κB plays a protumorigenic cell autonomous role only in inflammation driven HCC, which is in line with the known anti-apoptotic roles of NF-κB. So how does NF-κB inhibition facilitate tumorigenesis in the DEN model? We inhibited NF-κB at different periods in the DEN model and found that the suppressive role in DEN induced carcinogenesis is restricted to the very early period following DEN injection. Interestingly, at this time point, NF-κB activity leads to an increase in p21 dependent persistent cell cycle arrest following the DEN injection (see figure 1, 2a). This arrest is preceded by an NF-κB dependent increased ATM response. The hypothesis we are currently following is that NF-κB plays a unique role in modulating the DNA damage response (DDR). While it has been known for several years now that DDR can activate NF-κB, the consequences of this activation are not clear in this context. Our preliminary data shows that activation of NF-κB following Den results in improved DNA damage repair. We are currently seeking the specific NF-κB target genes that control the repair

process. Taken together, our data implies that the different effects of NF-κB in liver tumorigenesis take place at different phases of hepatocarcinogenesis: in the carcinogen model, at the very early point of carcinogen exposure while in the Mdr2-/- model, at a later period of tumorigenesis.

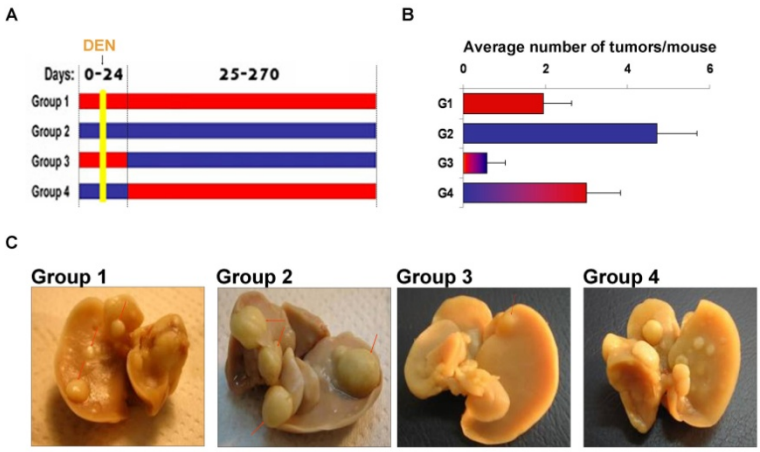

Figure1. **NF-κB suppresses hepatocarcinogenesis at the early phases of carcinogene induced HCC.**

a. experimental scheme: mice harboring a tetracycline regulatable hepatocyte specific I-κB-super repressor transgene were injected with diethylnitrosamin (DEN) at day 14 (yellow line). Red indicates tetracycline treatment (transgene inactive – hepatocyte NF-κB proficient); blue indicates no tetracycline (transgene active – hepatocyte NF-κB blocked).
b. inactivation of NF-κB at days 0-24 accelerated tumorigenesis, while its inactivation at later stages suppresses tumorigenesis.
c. representative pictures of tumors from the indicated groups. Taken together, this data suggests that NF-κB suppresses the early phases of DEN induced hepatocarcinogenesis.

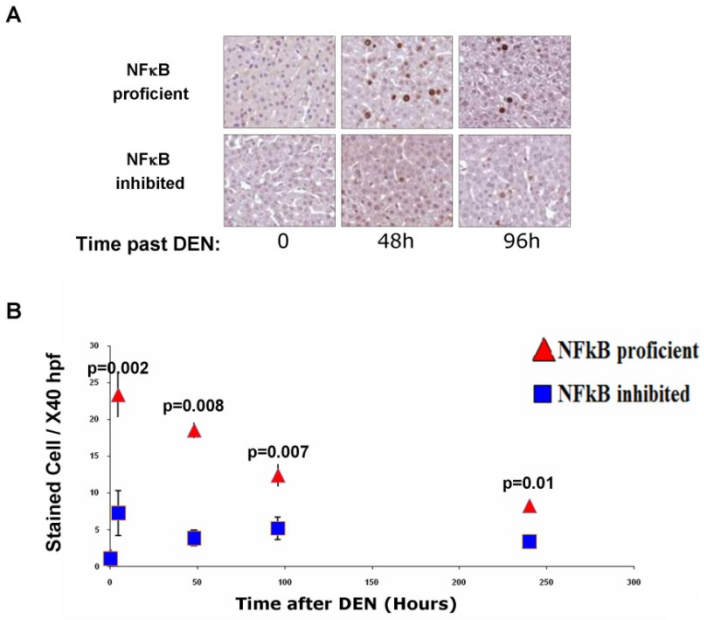

Figure 2. **NF-κB modulates the DNA damage response**.

a. hepatocyte NF-κB proficient and inhibited mice (see legend to figure 1 for experimental detail) were injected with DEN and sacrificed at the indicated times post treatment. Immunostaining for gH2Ax was performed as an indicator of activation of DDR. Shown are representative photomicrographs.
b. the number of positively stained nuclei for gH2Ax was assessed using automated image analysis. Note that while the activation is much more efficient in the NF-κB proficient group, the slope of the curve is higher in the same group, indicating more rapid repair.