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Meeting report

Ataxia-telangiectasia, ATM and genomic stability: maintaining a delicate balance
Two international workshops on ataxia-telangiectasia, related disorders and the ATM protein

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1. Introduction

Genetic alterations promoting the development of cancer occur in somatic cells or are inherited. Germline mutations in specific genes may lead to genetic predisposition to cancer which will be expressed as overt malignancy following additional somatic events. Genetic disorders involving cancer predisposition therefore flag cellular functions that guard cells from embarking on the road to neoplasia [1]. Naturally, the protein products of several tumor suppressor genes are directly involved in cell cycle control and cellular growth. A second group of these genes encodes proteins involved in maintaining genome integrity and stability [2]. In addition to various DNA repair enzymes, this group includes an emerging class of proteins that signal the presence of structural alterations in the DNA to systems controlling cellular proliferation. Such alterations may represent DNA damage, or intermediates of normal DNA metabolism, such as maturation of the immune system genes or meiotic recombination. In both cases, a temporary slow down of cell cycle progression may be needed while these structures are processed and genome integrity is restored.

The human autosomal recessive disorder ataxia-telangiectasia (A-T) appears to represent such a function [3,4]. A-T patients exhibit cerebellar degeneration, telangiectasia (dilated blood vessels) in the eyes or facial skin, thymic degeneration, immunodeficiency, premature aging, gonadal dysgenesis, predisposition to lymphoid malignancies, and acute radiation sensitivity. At the cellular level, prominent features are premature senescence, chromosomal instability, telomere shortening, high mitotic recombination, extreme sensitivity to ionizing radiation and radiomimetic chemicals, defective activation of cell cycle checkpoints by these agents, and defects in various signal transduction pathways which are normally induced by genotoxic stress. The responsible gene, ATM, was identified using a positional cloning approach [5,6], and its product seems indeed to be a multifunctional protein involved in a wide range of cellular processes [3,4,7,8].

The functional, genetic and clinical aspects of A-T and the ATM protein were the focus of two recent international workshops[1,2]. Additional discussions concerned another genomic instability disorder, the

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Nijmegen breakage syndrome (NBS), which shares some features with A-T. Patients with this rare disorder exhibit immunodeficiency, radiation sensitivity, chromosomal instability and cancer predisposition, but do not show cerebellar deterioration and telangiectasia. Additional features are microcephaly and occasionally mental retardation. While genomic instability and cancer predisposition may be more pronounced in NBS than in A-T, the cellular phenotypes of the two disorders are very similar [4,9].

A-T and NBS highlight one, or possibly two separate physiological pathways tying together genomic instability, radiation sensitivity and cancer predisposition. These pathways can be understood by elucidating the biology of the relevant proteins. While efforts to identify the NBS gene are in progress, information on ATM is rapidly accumulating, revealing the versatility of this multifaceted molecule.

2. The ATM protein

ATM is a predominantly nuclear protein of 370 kDa with a carboxy-terminal region resembling the catalytic domain of phosphatidylinositol 3-kinases (PI3K). ATM belongs to a family of PI3K-related proteins, identified in various organisms, which are involved in maintaining telomere length, cellular responses to genotoxic stress, mitotic and meiotic stability, and cell cycle progression [7]. A notable member of this family is the catalytic subunit of DNA-dependent protein kinase (DNA-PK), which is thought to be involved in DNA strand break repair and in signalling the presence of DNA discontinuities to various regulatory systems [10].

The unusually large open reading frame encoding ATM has made the production of a recombinant protein a technical challenge. Yael Ziv (Tel Aviv University) and Martin Lavin (Queensland Institute of Medical Research) reported the successful production of biologically active recombinant ATM whose expression corrects many aspects of the cellular A-T phenotype. Furthermore, full-length or partial ATM antisense RNA confers non-A-T cells with the disease features (Philip Chen, Queensland Institute of Medical Research; Nancy Uhrhammer, Yale University), indicating that absence of the protein is indeed responsible for this phenotype. Interestingly, Susan Morgan (Johns Hopkins University) and Tej Pandita (Columbia University) reported that ectopic expression of only the PI3K-related domain of ATM also complemented many features of the A-T phenotype, including telomere shortening. This is in spite of the fact that a similar fragment transfected into human cells by Kevin Brown (National Human Genome Research Institute) did not enter the nucleus.

In spite of the homology to lipid kinases, several members of the PI3K-related family have been shown to be serine/threonine protein kinases, e.g., DNA-PK. ATM is, thus, expected be a protein kinase as well. Protocols for purification of ATM to apparent homogeneity were reported by Nick Lakin (University of Cambridge) and Susan Lees-Miller (University of Calgary). While some ATM preparations showed no kinase activity, others contained additional kinases that co-purified with ATM. An associated protein kinase activity was observed in immune complexes obtained with anti-ATM antibodies ([11,12]; Richard Paules, NIEHS; Jann Sarkaria, Mayo Clinic). It should be noted, however, that these experiments lack an essential negative control, a 'kinase-dead' ATM. Such a control would allow one to know whether the ATM-associated kinase is the product of an associated protein or of ATM, itself. The great majority of A-T cells, which are routinely used as negative controls, have no ATM at all due to the nature of A-T mutations (see below). Therefore, extracts of these cells cannot be used to rule out co-purification or co-precipitation of other kinases with ATM immunoprecipitated from normal cellular extracts.

3. Clues to ATM's functions: tying together DNA damage processing and meiotic recombination

Elucidation ATM's involvement in cellular metabolism is based on fine characterization of the organismal and cellular A-T phenotype in humans and Atm-deficient mice, and identification of the activity and interactions of this protein. Yossi Shiloh (Tel Aviv University) noted the dual nature of ATM reflected in the biology of A-T cells. Unrepaired chromatid breaks, clonal translocations involving the immune system genes, and imperfect in vitro repair of DNA strand breaks by A-T cellular extracts all point

to a defect in the processing of specific types of DNA discontinuities. On the other hand, the defects in activating a variety of signal transduction pathways, most notably cell cycle checkpoints, also point to ATM as a sensor and signaler of DNA structural alterations to numerous cellular systems.

Evidence of ATM's involvement in processing of DNA damage in somatic cells was provided by Musbacher Dar (Georgetown University), who described defective illegitimate recombinational repair of double strand breaks in A-T cells. Akira Tachibana (Radiation Biology Center, Kyoto, Japan) reported misrejoining of double-strand breaks in vitro by A-T extracts, and Kouichi Tatsumi (National Institute of Radiological Sciences, Chiba, Japan) noted the excessive level of deletion mutations in irradiated A-T cells.

Parallel to these observations, direct physical association of ATM with DNA and chromatin were clearly demonstrated in meiotic cells. The involvement of ATM in the meiotic process, most probably in recombination, is highlighted by the complete sterility of Atm-deficient mice, which results from abnormal synapsis and chromosomal fragmentation ([13,14]; Tony Wynshaw-Boris, National Human Genome Research Institute). Annemieke Plug and Terry Ashley (Yale University) showed that in mice, Atm binds to meiotic chromosomes along their synapsed portions, where, together with other proteins, it becomes a component of structures called recombination nodules. Fragmentation of meiotic chromosomes in Atm-deficient mice indeed occurs at the sites of these structures. These observations set the stage for demonstrating the interaction of ATM with other proteins involved both in damage recognition and meiotic recombination (see below), and highlighted a second aspect of ATM function: maintaining genome stability in the context of both DNA damage and meiotic recombination.

Martin Lavin described the signaling role of ATM reflected in the defective activation of several stress response pathways in A-T cells. The best documented defect is retarded stabilization of the p53 protein following treatment with ionizing radiation or radiomimetic drugs. p53 is a central player in several cell cycle checkpoints, particularly the one at G1/S, since it enhances the production of the p21 protein which, in turn, inhibits several cyclin/Cdk complexes responsible for cell cycle progression. It is clear, however, that while the checkpoint defects may be involved in genomic instability of A-T cells, they have minimal or no responsibility for their radiosensitivity, and the acute cytotoxic effect of ionizing radiation on A-T cells should be attributed to other mechanisms. Steve Meyn (Yale University) offered an integrative model for ATM function based on sensing double-strand breaks (resulting from either damage or recombination), followed by activation of a signal transduction network coordinating cell cycle checkpoints and additional pathways. Galit Rotman (Tel Aviv University) pointed out a possible role of continuous oxidative stress in the A-T phenotype, and demonstrated a chronic elevation of various stress-induced proteins in A-T cells, which was reduced by treatment with antioxidants. Tenous oxidative stress may account, in part, for the progression of A-T, particularly in the nervous system.

4. ATM's physical and functional interactions

It is assumed that the PI3K-related carboxy-terminal region spanning about 10% of the ATM molecule harbors the catalytic kinase domain. What does the rest of this big molecule do? That portion may explain the extreme pleiotropic effect of its absence, since it probably harbors sites for interaction with other proteins. But, functional interaction between ATM and other proteins may not require their direct physical association. Interestingly, ATM seems to interact with some proteins at several levels at once, modulating their production and physically associating with them.

One of the first candidates tested for possible interaction with ATM is the non-receptor nuclear tyrosine kinase, c-Abl [15,16]. Kum Kum Khanna (Queensland Institute of Medical Research) and Jean Wang (University of California, San Diego) summarized these results. c-Abl's phosphorylation in response to radiation damage activates its tyrosine kinase activity. Among the substrates for this activity are the C-terminal repeated domain (CTD) of RNA polymerase II, and the Jun N-terminal kinase (JNK) whose activity is directed at the c-Jun protein. These responses are defective in A-T cells, and, hence, appear to be ATM-dependent. c-Abl binds constitu-

tively to a proline-rich 10 amino acid ATM motif through its SH3 domain. Immune complexes containing the PI3K fragment of ATM exhibit an associated kinase activity which phosphorylates c-Abl on Ser^{465}, although phosphorylation of c-Abl by full-length ATM has not been demonstrated. An ATM/c-Abl interaction may be the first model of a signal transduction pathway initiated by phosphorylation of an effector protein by ATM.

Considerable attention is given to the physical and functional interactions of ATM with proteins known to be involved in the cell cycle checkpoints defective in A-T cells. Martin Lavin and Padmini Kedar (Queensland Institute of Medical Research) presented data indicating that ATM not only modulates the cellular levels of p53 and consequently p21, but also interacts physically with both of them. It is not clear, however, whether these proteins are direct substrates of ATM kinase activity. In view of the recent observation of radiation-induced phosphorylation of p53, which interferes with its interaction with the mdm2 protein that targets p53 for degradation [17,18], ATM appears to be an attractive candidate for the kinase responsible for this phosphorylation.

A central role in the G2/M checkpoint is played by the Chk1 kinase, initially identified in the fission yeast. Chk1 is phosphorylated following DNA damage and inactivates the Cdc25 phosphatase. Inactive Cdc25 is unable to turn on, by dephosphorylation, the cyclin-dependent kinase $p34^{Cdc2}$, which propels the progression from G2 to mitosis. Chk1's activation in the fission yeast is dependent on Rad3p, a member of the PI3K family, which might be the kinase responsible for its phosphorylation. This pathway is conserved through evolution, so human Chk1 might act downstream of ATM or ATR, the closest human homolog of Rad3p. The human and mouse Chk1 proteins were recently cloned by several groups [19,20], one of which was represented by Merl Hoekstra and Gail Flaggs (ICOS). Human Chk1 indeed phosphorylates the Cdc25C phosphatase [19], but there is no evidence yet of its direct phosphorylation by ATM. Martin Lavin showed that ectopic expression of Chk1 in A-T cells selectively corrects the G2/M checkpoint defect.

Merl Hoekstra and Terry Ashley demonstrated an interesting collaboration between Atm and Chk1 in mouse meiosis [20]. Both the Atm and Atr protein associate with pairing meiotic chromosomes in the mouse, with preference of Atr to unpaired (asynapsed) chromosomal axes while Atm binds to synapsed chromosomal axes [12]. Chk1 follows them and binds to both synapsed and asynapsed portions of meiotic chromosomes. Interestingly, Chk1 levels in testes of Atm-deficient mice are severely reduced. It is not clear whether this phenomenon results from reduction in transcription or protein instability. It is becoming clear, however, that Chk1 operates downstream of Atm, possibly at recombination nodules.

Annemieke Plug and Terry Ashley showed that another component of the recombination nodules in mice is replication protein A (Rpa), known to be involved in processing and signaling DNA damage [21]. In Atm-deficient mice, Rpa remains attached to the ends of the abnormal chromosomal fragments formed at the sites of recombination nodules.

ATM thus joins a growing list of proteins known to be involved in processing both genotoxic damage and meiotic recombination intermediates. A well-known member of this list is Rad51, a homolog of the bacterial RecA protein. Indeed, Carrolee Barlow reported defective assembly of Rad51 foci on unpaired axial elements in spermatocytes of Atm-deficient mice [22]. Eva Lee (University of Texas) presented evidence for the involvement of the human Rad51 protein in a complex shared also by ATM and c-Abl in somatic cells. c-Abl was found to interact with and phosphorylate Rad51 in vitro, and this phosphorylation enhanced the formation of a complex between Rad51 and Rad52, which operates in recombination and repair. This process is induced in wild-type cells by ionizing radiation, but fails to be activated in A-T cells or in c-Abl-deficient cells. These observations point to the role of ATM and c-Abl in a signaling pathway that promotes the assembly of recombinational repair complexes.

A link between cancer predisposition, response to genotoxic stress and association with meiotic chromosomes is also exhibited by the BRCA1 protein, the product of one of the major culprits in genetic predisposition to breast cancer. BRCA1 is known to interact with Rad51 both during the S-phase in somatic cells, where they take part in the same discrete nuclear foci, and along axial elements of human meiotic chromosomes [23,24]. In mice, the Brca1 protein

associates with meiotic chromosomes in a fashion similar to Atr. Merl Hoekstra presented evidence of physical association between Brca1 and Atr as well as phosphorylation of the Brca1 protein by Atr in vitro, but corroborated previous results showing that damage-induced phosphorylation of Brca1 is independent of DNA-PK and ATM.

A powerful tool for the study of functional interaction between different proteins is phenotypic analysis of double mutant mice. Christoph Westphal (Harvard Medical School), Carrolee Barlow and Yang Xu discussed the organismal and cellular phenotype of mice deficient for both Atm and p53 [22,25–27]. The data indicate that Atm is selectively involved in some, but not all, p53-mediated processes. Thus, while absence of p53 does not correct the G1/S checkpoint defect of Atm-deficient mouse embryo fibroblasts, it does relieve their growth arrest in early passage levels. Radiosensitivity is similar in Atm/p53-deficient cells, but tumor formation is significantly faster in the double knockout animals, than in those deficient for Atm only.

An important, tissue-specific and p53-mediated response to genotoxic stress is apoptosis. Barlow and colleagues noted normal radiation-induced apoptosis and Bax induction in the thymus of Atm −/− mice, which was suppressed when p53 was inactivated as well; they concluded that this response is controlled by p53 in an Atm- independent manner. Interestingly, Westphal and colleagues observed partial resistance to radiation-induced apoptosis in Atm-knockout thymocytes, which was augmented when p53 was also inactivated. They concluded that Atm- and p53-mediated apoptotic pathways are not completely separate. On the meiotic front, inactivation of p53 or p21 in addition to Atm partially rescued the early arrest of the process (Barlow). Rad51 association with meiotic chromosomes remained defective. It seems, therefore, that Atm provides a checkpoint necessary for Rad51 assembly on axial elements.

The ATM protein is thus involved in a number of separate, but sometimes interacting signal cascades involving responses to DNA discontinuities. How can we account for the fact that complete deficiency of a protein apparently involved in functions critical to cellular survival results in a slowly progressing disease rather than embryonic death? One possible answer is 'redundancy'. It is highly likely that ATM functions are shared by several proteins (some of which may be other members of the PI3K-related family), and the complete lack of ATM leads merely to reduced efficiency, but not complete loss of these functions. A vivid example of this principle was provided by Eberhard Fritz (Yale University) who showed that overexpression of the budding yeast protein, TEL1p, in A-T cells complements several features of their phenotype. Interestingly, this member of the PI3K family is responsible in yeast cells primarily for maintaining telomere length. Another explanation is that the consequences of ATM absence, such as accumulation of DNA damage, are more critical for the well-being of specific types of cells, e.g. Purkinje cells and thymocytes.

5. Molecular genetics of A-T

Can we identify critical regions in the ATM molecule by localizing A-T mutations? Clinical signs and characteristics of the cellular phenotype vary considerably in large series of A-T patients (Ozden Sanal, Hacettepe University, Ankara). The majority of patients are considered 'typical', while those with milder phenotypic features are designated 'variants'. An enormous repertoire of mutations characterizes the typical patients, but their outcome at the protein level is the same in the vast majority of cases. These mutations lead to truncations or large deletions in the ATM protein, leaving the patients with no ATM at all (Richard Gatti, UCLA School of Medicine; Malcolm Taylor, University of Birmingham; Thilo Doerk, Institute of Human Genetics, Hannover; Anne-Lise Borresen-Dale, Norwegian Radium Institute, Oslo; Annegien Broeks, Netherlands Cancer Institute, Amsterdam; Sabrina Prudente, La Sapienza University, Rome). Mutations in many of the variants leave a residual amount of the ATM protein (Malcolm Taylor; Anat Bar-Shira, Tel Aviv University).

6. Are A-T carriers predisposed to cancer?

This highly debated question is being extensively explored using ATM-derived reagents. The epide-

miological evidence for increased frequency of malignancies, particularly breast cancer, among blood relatives of A-T patients was reviewed by Michael Swift (New York Medical College). Several groups are screening cohorts of cancer patients for germ-line ATM mutations, with special emphasis on breast cancer patients. A large series of early-onset breast cancer patients was presented by Michael FitzGerald (Massachusetts General Hospital) [28], and smaller ones by the German ATM Consortium, Annegiem Broeks, Louise Izatt (Guy's Hospital, London), Nicolas Janin (Institut Gustave Roussy, Paris), Sandra Wolman (Uniformed Services University of the Health Sciences, Bethesda) and Regina Bendix (Institute of Human Genetics, Hannover). Results to date do not indicate any significant increase in ATM carrier frequency among these patients. Several researchers looked at cancer patients exhibiting an adverse reaction to radiotherapy. A-T heterozygous cell lines show moderate radiosensitivity, but there are no in vivo correlates of this phenomenon. Jonathan Ramsay (Queensland Institute of Medical Research) and Janet Hall (International Agency for Research on Cancer, Lyon) did not observe elevated frequencies of A-T carriers among several scores of radiosensitive cancer patients.

An alternative approach to the problem is based on identification of carriers in extended A-T families coupled with examination of their medical history. Preliminary results from on-going studies in Italy (Luciana Chessa, La Sapienza University) do not indicate a higher incidence of cancer among identified carriers, but two other studies did find a possible elevation of the risk for cancer among these individuals. Dominique Stoppa-Lyonnet (Institut Curie, Paris) identified 240 carriers among 1429 relatives of A-T patients, and observed among them significant elevation of breast cancer compared to non-carriers. The overall frequency of cancers did not differ, however, from that of the control group. Anne-Lise Borresen-Dale noticed in 290 second- and third-degree relatives of Norwegian A-T patients a relative risk of 1.67 for all types of cancer, and 3.38 for breast cancer in females.

These results still do not permit definite conclusions about this issue. It does appear, though, that the effect of heterozygosity for A-T mutations on cancer predisposition is relatively small, certainly less than that of the BRCA1 and BRCA2 genes.

7. ATM as a tumor suppressor gene

New results about the role of ATM mutations in cancer came from studies on ATM mutations identified in tumor tissues. The classical model of tumor suppressor action is based on germ-line mutations that lead to predisposition to cancer among their carriers, with further somatic events leading to malignancy. In many tumors, the chromosomal region harboring ATM, 11q22-23, is indeed involved in the loss of heterozygosity (LOH) typical of tumor suppressor sites (Anne-Lise Borresen-Dale; Pascale Rio, Centre Jean Perrin; Maria Worsham, Henry Ford Hospital). It remains to be seen, however, whether ATM is the target of LOH in this region. In view of the data on cancer frequency among A-T heterozygotes, ATM may not follow the classical mode of action of tumor suppressor genes. On the other hand, the acute cancer predisposition of A-T patients might signify a role for ATM as a gatekeeper in certain cancers. One of the common malignancies among A-T patients (but not carriers) is T-prolymphocytic leukemia (T-PLL), which is very rare in the general population. Four groups presented evidence of inactivation of both ATM copies in more than 50% of T-PLL tumors in non-A-T patients (Igor Vorechovsky, Karolinska Institute; Martin Yuille, Royal Marsden Hospital, London; Claudia Schaffner, German Cancer Research Center, Heidelberg; and Dominique Stoppa-Lyonnet; see also [29–31]). The inactivating lesions ranged from rearrangements to point mutations, which tend to cluster at the PI3K region. Igor Vorechovsky identified such mutations in 2/32 B-cell non-Hodgkin's lymphomas as well.

Interestingly, there is no excess of germ-line carriers of A-T mutations in this group, which means that both ATM-inactivating events occur in somatic cells. In this respect, ATM belongs to a unique class of tumor suppressor genes that serve as gatekeepers in specific tumors, and bring about malignancy only in germ-line homozygotes, or by purely somatic events in homozygous normal individuals. It is difficult to explain why A-T carriers are not prone to T-

PLL. Further understanding of the biology of this particular tumor may help explain this paradox.

8. The Nijmegen breakage syndrome (NBS)

NBS, previously regarded as an 'A-T variant', is now clearly established as a separate clinical and genetic entity following localization of the NBS locus on chromosome 8q21 ([32]; Kathrin Saar, Max-Delbrueck Center, and Karl Sperling, Human Genetics Institute, Berlin). Introduction of a normal chromosome 8 into NBS cells indeed complements the disease phenotype (Kenshi Komatsu, Hiroshima University; W.J.I. Overkamp, Leiden University). At the same time, the cellular phenotype of NBS cells has been compared to that of A-T cells in an effort to determine functional relationships between the products of the two disease genes. For example, the response to genotoxic stress is very similar in A-T and NBS cells, but results sometimes differ among laboratories on certain parameters (Janet Hall; Cordula Kirschgessner, Stanford University). NBS may represent a pathway operating parallel to that of ATM, or a component of the ATM pathway. The latter possibility could account for separate ATM functions related to stress responses on one hand and the cerebellar defect, which is not found in NBS, on the other. An intense positional cloning effort is jointly being made by the groups of Karl Sperling and Pat Concannon (Virginia Mason Research Center, Seattle) in search of the NBS gene whose identification appears to be imminent.

9. What's next?

Work on the ATM protein is branching out in several directions. While intense efforts are underway to purify ATM in an active form and identify its substrates, attempts are being made to find additional proteins that take part in ATM complexes, and to identify genes whose expression is influenced by the presence of ATM. Atm −/− mice will continue to provide valuable opportunities to study the interaction between ATM and other genes as well as the pathological processes occurring in affected tissues. Particularly intriguing are the highly specific involvement of ATM in the causation of a particular tumor, T-PLL, and the yet unanswered question of cancer predisposition of A-T carriers. The isolation of the NBS gene should contribute important insights into the mechanisms responsible for the clinical and cellular phenotypes of A-T and NBS.

This expanding field has attracted the attention of scientists from diverse fields of biomedical research, and this seems likely to continue.

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