

A20 takes on tumors: tumor suppression by an ubiquitin-editing enzyme

Barbara A. Malynn and Averil Ma

Many B cell cancers are characterized in part by the dysregulation of the NF- κ B signaling pathway. A new study identifies somatic mutations in *TNFAIP3*, the gene encoding the NF- κ B inhibitor A20, in Hodgkin lymphomas and primary mediastinal lymphomas. These data reveal the role of A20 as a tumor suppressor protein.

Lymphomas are the fifth most common of human malignancies; the chance of developing lymphoma during one's lifetime is \sim 2%. The vast majority of lymphomas are B cell lineage derived and can be characterized as Hodgkin or non-Hodgkin lymphomas. Both classes can be further subtyped based upon their biological properties, including differentiation stage, clinical pathology, and the response to therapeutic treatment (1). Many important clues to lymphoma pathogenesis have come from cytogenetic and genetic analyses of somatic mutations (e.g., translocations and gene deletions), as well as gene expression profiling (2). Mutations in NF- κ B and JAK/STAT signaling proteins cause defects in B cell survival and proliferation and contribute to lymphomagenesis (3). On page 981 of this issue, Schmitz et al. examine whether mutations in the *TNFAIP3* gene contribute to cellular transformation in human B cell lymphomas (4). Their data show that a large number of Hodgkin lymphomas and primary mediastinal lymphomas contain bi-allelic mutations of *TNFAIP3* that result in loss of the A20 protein. Other recent studies have also found *TNFAIP3* mutations in marginal zone B cell lymphomas (5, 6). These and future studies of ubiquitin modifiers may reveal new

directions for the discovery of anticancer therapies.

A20 keeps the peace

A20 was first identified in 1990 as a TNF-induced molecule that restricts TNF-triggered NF- κ B signaling (7). Its widespread expression and potent anti-inflammatory functions were unveiled when A20-deficient mice (A20^{-/-}) were found to exhibit severe spontaneous multiorgan inflammation, cachexia, and premature death (8). Epistasis experiments revealed that genetic deletion of TNF or TNF receptor 1 (TNFR1) in A20-deficient mice did not rescue the mice from premature death, demonstrating that A20 regulates proinflammatory signals in addition to TNF (9). We now know that A20 also restricts NF- κ B signaling in response to stimulation of the TLR and NOD pathways (9, 10). A20 regulates innate immune homeostasis independently of adaptive immune cells, as A20^{-/-} RAG-1^{-/-} mice lacking mature T and B cells develop severe spontaneous inflammation and perinatal death (8). The defects caused by A20 deficiency were ameliorated in mice that also lacked the TLR adaptor protein MyD88 and in mice treated with antibiotics to deplete commensal intestinal bacteria (11). These results highlight the potentially proinflammatory nature of homeostatic MyD88-dependent signals, as well as A20's critical role in restricting these signals and maintaining immune homeostasis.

The mechanics of A20 action

The biochemical mechanisms by which A20 restricts NF- κ B signaling are unique and complex. The A20 protein contains an ovarian tumor (OTU) domain at the N terminus and seven zinc finger (ZF) domains at the C terminus. The OTU domain functions as a deubiquitinating (DUB) enzyme to remove activating lysine-63 (K63)-linked polyubiquitin chains from receptor-interacting protein (RIP), an essential mediator of the proximal TNFR1 signaling complex. The A20 ZF domain functions as an E3 ubiquitin ligase, adding K48-linked polyubiquitin chains to RIP1 and targeting the protein for proteasomal degradation. Hence, A20 appears to be a dual function enzyme that adds and subtracts ubiquitin moieties to deactivate and degrade RIP (12). In addition to

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regulating TNF-induced RIP ubiquitylation, A20 restricts TLR-induced NF- κ B signals by deubiquitylating the E3 ligase TRAF6 (9, 11). A20 also dampens NOD1- and NOD2-induced NF- κ B signals by deubiquitylating RIP2, possibly preventing it from recruiting IKK to the signaling complex (10). Hence, like other DUBs and E3 ligases, A20 regulates the ubiquitylation of multiple protein targets.

B.A.M. and A.M. are at Program in Biomedical Sciences, Program in Biological Sciences, Department of Medicine, University of California, San Francisco, San Francisco, CA 94143.

CORRESPONDENCE

A.M.: averil.ma@ucsf.edu

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A20 also binds to the E3 ligases TRAF1, TRAF2, TRAF6, Itch, and RNF11, as well as to the adaptor protein TXBP1 and the ubiquitin sensor ABIN-1 (13–17). These proteins collaborate with A20 to regulate NF- κ B and MAP kinase signaling cascades and TNF-induced cell death. How this ubiquitin-dependent, multiprotein complex functions, how it targets specific signaling cascades, and whether distinct subsets of this complex preferentially regulate different signaling proteins are among the questions that remain to be answered.

A20 also inhibits programmed cell death (PCD) (8, 18). Increased susceptibility to PCD in A20-deficient cells is not caused by deficiencies in the expression of NF- κ B-induced survival proteins, as these cells have exaggerated NF- κ B signaling and normal expression of NF- κ B survival proteins, yet are more susceptible to TNF-induced PCD (8). Because limiting the lifespan of activated immune cells helps restrain inflammatory responses, the antiinflammatory and antiapoptotic functions of A20 impart dichotomous regulatory influences on immune homeostasis.

A20 in lymphocytes

Although most studies investigating the function of A20 have focused on innate immune signals, A20 is constitutively expressed and can be further induced in both T and B cells (7, 8, 19). However, the protein's function in lymphocytes is poorly understood. In T cells, the mucosal-associated lymphoid tumor-associated (MALT1) protein cleaves A20 during TCR activation, which is one mechanism by which MALT1 promotes TCR-induced NF- κ B activation (20). BCR stimulation of B lymphoma cell lines also leads to A20 cleavage. However, the physiological role of A20 and its cleavage in T and B cells is unclear. Mature B cells are regulated by NF- κ B signals triggered by the BCR, BAFF, and CD40. Because these signaling cascades may share some of the same ubiquitin-dependent proximate signaling molecules used by TNF and TLR ligands (e.g., TRAF2, TRAF6), it

is possible that A20 may also regulate B cell homeostasis and activation.

The antiinflammatory and antiapoptotic functions of A20 impart dichotomous regulatory influences on immune homeostasis

A20's cancer connection

A20-deficient cells exhibit prolonged NF- κ B signaling in response to a variety of innate immune ligands, and chronic NF- κ B activation has been associated with lymphomas (3). Genetic analyses have identified deletions in chromosome 6q that are associated with Hodgkin lymphoma and other subtypes of B cell lymphomas (e.g., marginal zone lymphomas, MALT lymphomas, and diffuse large B cell lymphomas). As the *TNFAIP3* gene is located at 6q23, Schmitz et al. (4) proposed that A20 might be pathogenetic in these human cancers. The authors focused their molecular analysis on primary mediastinal B cell lymphomas (PMBLs) and classical Hodgkin lymphoma (cHL), as the transcriptional profiles of these tumors are similar.

Remarkably, they found that biallelic somatic mutations in *TNFAIP3* were found in 16/36 cHLs and 5/14 PMBLs. Reconstitution of A20 expression induced cell death in A20 mutant lymphoma cell lines, but not in A20-sufficient lymphoma cells. These findings provide direct evidence that A20 is a tumor suppressor gene that is mutated with high frequency in these human lymphomas.

Schmitz et al. also make the intriguing observation that A20 mutations are found in 14/20 (70%) EBV-negative cHL compared with only 2/16 (12.5%) EBV-positive lymphomas, suggesting that EBV-dependent transformation perturbs B cell signaling in a fashion similar to A20 deletion. Indeed, earlier studies suggested that the EBV latent membrane protein 1 may mimic CD40 signaling by interacting with TRAFs,

and perhaps A20, thereby inducing constitutive NF- κ B signaling (21, 22). Although the precise biochemical relationships between EBV proteins and A20 are unclear, the genetic epistasis unveiled by Schmitz et al. may provide intriguing clues about B cell lymphomagenesis.

Most of the *TNFAIP3* mutations found in Hodgkin and mediastinal lymphomas were nonsense or frameshift mutations that prevented production of full-length A20 protein (4). Combined with the observation that both alleles were mutated in the tumor samples, the common pathogenetic mechanism of A20 in lymphoma development is likely a loss of protein function. Mutations were found throughout the coding sequence, and some mutations had the potential to encode partial proteins. As partial A20 proteins containing only the N-terminal OTU domain have been shown to retain deubiquitinating activity (9), the E3 ligase activity of A20 may be critical for its tumor suppressor function. It is also intriguing that six of the seven replacement mutations observed in both cHL and PMBL were clustered in the terminal zinc finger domains ZF6 and ZF7, which are thought to be largely dispensable for A20's E3 ligase activity (12). This suggests that subtle reductions in E3 ligase activity that may result from these mutations can be tumorigenic. Alternatively, previously unappreciated functions of A20 may be harbored in these domains.

The common pathogenetic mechanism of A20 in lymphoma development is likely a loss of protein function

Autoimmunity–cancer link?

Several chronic autoimmune diseases are associated with increased risk of developing lymphoma (23). Recent genetic studies in humans revealed several polymorphisms in the *TNFAIP3* gene region as risk alleles for the development of systemic lupus erythematosus

(SLE), rheumatoid arthritis, psoriasis, and celiac disease (24–29). While only one missense coding allele has been identified thus far (in SLE), the well-established antiinflammatory functions of A20 in mice suggest that hypomorphic function or expression of A20 may contribute to the pathophysiology of these human conditions (24). Collectively, these findings suggest that subtle germline *TNFAIP3* hypomorphisms may predispose individuals to chronic autoimmune diseases, whereas gross A20 deficiencies occurring somatically may contribute to lymphoma. In this context, it will be revealing to further analyze *TNFAIP3* polymorphisms in autoimmune patients and determine whether there is an association between certain polymorphisms and lymphoma risk. Considered from another perspective, the severe pathogenetic consequences of A20 deficiency may mean that deletional germline mutations of A20 are unlikely to be detected in the human population. These studies along with the results presented by Schmitz et al. (4) and others (5, 6) suggest that A20 may be a prevalent suppressor of both autoimmune disease and lymphoma in human patients, thereby providing a critical molecular link between chronic inflammation and cancer.

Questions for the future

As in most malignancies, the generation of lymphomas is likely to involve somatic mutations of multiple genes. Identifying oncogenic events that cooperate with *TNFAIP3* deletions in lymphomagenesis can now be tested in mice bearing mutations in known oncogenes or tumor suppressor genes. In this context, the rampant inflammation and markedly shortened lifespan of A20-deficient mice have largely precluded efforts to monitor these animals for spontaneous tumor development. Lineage-specific deletions of A20 may greatly facilitate these studies. In this regard, it will be particularly interesting to determine how A20 intrinsically regulates B cell functions by analyzing mice lacking A20 specifically in B cells.

Many questions also remain unresolved regarding the role of A20 in pro-

moting and/or blocking cell death. Although expression of A20 triggered PCD in A20 mutant lymphomas, A20 also acts as a survival protein, as it protects most cells from TNF-induced PCD (7, 8). This feature sets A20 apart from most other known tumor suppressors (e.g., p53), which are proapoptotic, antiproliferative, or both. This dichotomy of A20 function may provide clues regarding secondary mutations that may collaborate with A20 mutations to promote tumorigenesis. It is also important to note that A20 may regulate JNK signaling pathways via its interactions with TRAF2 (13, 14). Hence, regulation of the NF- κ B pathway may not be A20's sole function in tumorigenesis.

A20 may be a prevalent suppressor of both autoimmune disease and lymphoma in human patients

The remarkably high prevalence of *TNFAIP3* mutations in these lymphomas suggests that A20 is a biomedically important tumor suppressor. In addition, A20 and NF- κ B are expressed in virtually all cell types; thus, A20 deletions may be involved in the pathogenesis of other lymphomas and/or other malignancies. The continuing identification of proteins that interact with A20 may also reveal other candidate tumor suppressors.

Finally, akin to the dysregulation of phosphorylation-dependent signaling events in cancer pathogenesis, the identification of ubiquitin-dependent events that contribute to transformation opens up new opportunities for tumor classifications and novel therapeutics. Dissecting the precise biochemical mechanisms of A20 function, e.g., how proximate signaling molecules are ubiquitinated, how ubiquitin-dependent sensors and enzymes interact, and what structural motifs determine ubiquitin sensing, may lead to molecular insights that spawn small molecule inhibitors of ubiquitin-dependent protooncogenic signals.

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