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Review

In situ detection of APRIL-rich niches for plasma-cell survival and their contribution to B-cell lymphoma development

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Summary. A proliferation inducing ligand (APRIL) is one of the most recently cloned members of the tumor necrosis factor (TNF) family. Early experiments implicated a pathophysiological role for APRIL in the promotion of solid tumors. Later, identification of APRIL receptors on B lymphocytes indicated a physiological role for APRIL in humoral responses. We have been able to generate antibodies that detect APRIL protein in human tissues. The study of in situ APRIL expression showed that APRIL mainly regulates late stages of B-cell humoral responses. It also provided evidence that APRIL may modulate tumor development in patients, but only for specific B-cell malignancies. Here, we will review to what extent fine characterization of in situ expression adds valuable information on APRIL (patho) physiological functions.

Key words: APRIL, Inflammation, Humoral immunity, B-cell neoplasia

Introduction

A proliferation inducing ligand (APRIL, TNFSF-13) is one of the most recently cloned members of the tumor necrosis factor (TNF) superfamily (Kelly et al., 2000) (Hahne et al., 1998). Like many members of the TNF superfamily, APRIL is a homotrimer with one ß-stranded extracellular TNF homology domain (THD). APRIL is initially produced as a type II transmembrane protein, which is released in the extracellular compartment after cleavage in the Golgi apparatus by furin-like proteases (Lopez-Fraga et al., 2001). The early cleavage in the biosynthetic pathway prevents cell surface expression.

APRIL was first identified as a factor, mostly autocrine, promoting solid tumor development (Hahne et al., 1998; Rennert et al., 2000; Roth et al., 2001). However, identification of APRIL receptors raised doubts as to this role. Indeed, APRIL has two canonical TNF receptors (TNF-R), the B-cell maturation antigen (BCMA, TNFRSF-17) and the transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI, TNFRSF-13b) (Kalled et al., 2005), which are restricted to the hematopoietic lineage. Expression of BCMA is exclusive to the B-cell lineage, while TACI expression has also been reported in T cells and macrophages, in addition to B cells. Expression of BCMA and TACI at the different stages of B-cell differentiation has been controversial due to the different antibodies used. However, it is now widely accepted that both receptors are expressed in B cells after antigen encounter with an expression of BCMA in germinal center B cells, memory B cells and plasma cells (PC), while TACI is expressed in memory B cells and PC (Mackay and Schneider, 2008). BCMA harbors one cysteine-rich TNF-R homology domain (TRHD), while TACI has two. Despite this difference, APRIL/TACI and APRIL/BCMA interactions are similar, since TACI uses only its TRHD most proximal to the membrane for ligand binding (Hymowitz et al., 2005). TACI signaling is versatile. It can either stimulate humoral immunity by delivering a signal for Ig switch to antigen-stimulated B cells, or have a negative impact by dampening B-cell activation upon antigen encounter (Salzer et al., 2007).

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The latter negative signaling may explain the unexpected occurrence of B-cell lymphoma in *TACI*-deficient mice (Seshasayee et al., 2003). In contrast, BCMA acts positively in respect to humoral immunity by giving a survival signal to PC (O'Connor et al., 2004). APRIL binds TACI and BCMA with a similar affinity, 11.8 nM and 16 nM respectively (Hymowitz et al., 2005). APRIL also interacts with heparan sulfate side chains of proteoglycans (HSPG) through a stretch of basic amino acids. This domain is present in the secreted product as it is located directly after the furin cleavage site in the protein sequence (Hendriks et al., 2005). HSPG also interact with TACI (Sakurai et al., 2007), rendering the interaction between the different ligands and receptors complex. Our current understanding of APRIL signaling is that HSPG serve as co-receptors that enable the signaling of the biologically inactive trimeric form of APRIL (Ingold et al., 2005). In addition, the direct interaction of HSPG with TACI activates the TACI signaling pathway (Sakurai et al., 2007). Hence, the result of APRIL signaling will depend on the APRIL-R phenotype of the responding cells, and can potentially be mechanistically and biologically very different from one cell to another.

APRIL, a cytokine detectable in human lymphoid organs

Early studies performed shortly after its cloning reported that APRIL is broadly expressed in tissues (Kelly et al., 2000). Later, APRIL mRNA expression was reported in hematopoietic cells from the myeloid lineage (MacLennan and Vinuesa, 2002), but also in megakaryocytes (Bonci et al., 2004), osteoclasts (Moreaux et al., 2005), and mesenchymal cells (Langat et al., 2008), as well as in a variety of tumor cell lines. Taken together, this indicates that APRIL mRNA is widely expressed.

Two APRIL-specific antibodies have been instrumental to study APRIL protein expression in situ (Schwaller et al., 2007). One recognizes the N-terminal APRIL fragment, referred to as APRIL stalk, which remains associated with the membrane after APRIL processing. Because the stalk fragment is stable, an anti-stalk antibody can be used to identify APRIL-producing cells. The second antibody recognizes the THD of APRIL, and is of interest because the full-length protein is not detected in producing cells by conventional immunohistochemistry. This is most likely due to the early processing of APRIL in the biosynthetic pathway (Lopez-Fraga et al., 2001). Hence, this second antibody detects only secreted APRIL. As a consequence, reactivities against the stalk fragment and the secreted form of APRIL are very different. The anti-stalk antibody results in a uniform intracellular staining of producing cells, while the antibody against the secreted product yields a characteristic pattern of dots that represents secreted APRIL bound to tissue HSPG. Figure 1 shows a scheme of APRIL staining classically observed in tissues, with APRIL-producing neutrophils identified by the presence of the stalk fragment in their cytoplasm, and secreted APRIL retained in the tissue by interaction with HSPG. Many TNF ligands harbor a protease cleavage site in their extracellular domain, leaving also a stalk fragment in the producing cells after processing. Hence, antibodies against the stalk of these different TNF ligands may be valuable to detect producing cells in situ.

In situ APRIL expression in lymphoid organs unravels APRIL functions

The antibody against APRIL stalk typically stains $CD15^+$ $elastase^+$ neutrophils in sections from normal lymphoid tissues (Huard et al., 2008). This implies that the APRIL mRNA observed in other myeloid cells and megakaryocytes in vitro does not lead to a sufficient production of the APRIL protein for detection by standard immunohistochemistry. For osteoclasts, it is not yet known whether they express a high level of APRIL in situ, as too few were present in the tissues we analyzed. Thus, neutrophils constitute the main cellular source of APRIL in situ. These cells are an excellent supply of secreted APRIL. Indeed, they do not retain the secreted product due to their lack of HSPG expression (Mhawech-Fauceglia et al., 2006), and, when activated, their release of elastase destroys adjacent HSPG (Watanabe et al., 1990), enabling APRIL to act at distance from its source. Although requiring confirmation by quantitative RT-PCR, APRIL

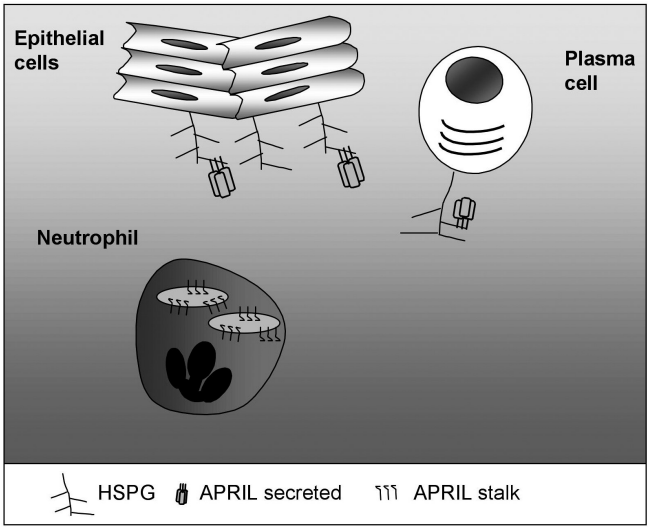

Fig. 1. Uncommon detection of APRIL compared to other cytokines in tissues. In tissues, the APRIL protein is produced by neutrophils. In these cells, APRIL processing by furin-like proteases leaves an intracellular stalk-fragment enabling the detection of APRIL-producing cells, while the soluble product is secreted. This soluble product is retained in tissues by HSPG, allowing its precise tissue localization.

production by neutrophils appears to be constitutive, since the stalk staining obtained for bone marrow, blood and tissue-infiltrating neutrophils is indistinguishable. A subset of epithelial (Huard et al., 2008) and endothelial cells (Schwaller et al., 2007) is also positive for the stalk fragment. Epithelial cells produce APRIL after toll-like receptor (TLR) stimulation (He et al., 2007), but it is presently not known what triggers APRIL production in endothelial cells. In contrast, we did not observe an expression of APRIL by dendritic cells as reported previously (Seyler et al., 2005; Xu et al., 2007). Since neutrophils are the main cellular source of APRIL in vivo, it implies that APRIL production is regulated by inflammation. At the level of lymphoid organs this statement holds, with the exception of the bone marrow and the spleen. In the bone marrow, APRIL production is constitutive, due to production early in the maturation process of granulocytes (Schwaller et al., 2007). In fact, there is no neutrophil that does not produce APRIL. The spleen is also a lymphoid organ showing constitutive production, due to the presence of a large number of APRIL-producing neutrophils in the red pulp and the marginal zone (unpublished observations). In lymph nodes and mucosa-associated lymphoid tissues (MALT), such as the tonsil, there is very little production of APRIL in the absence of infection. However, pathogen infection in the tonsil crypt induces a massive recruitment, chemokine-driven (Ebenfelt and Ivarsson, 2001), of APRIL-producing neutrophils (Huard et al., 2008). This production by neutrophils is complemented by the upregulation of APRIL production by epithelial cells upon TLR triggering. In another MALT, the small intestine, APRIL-producing neutrophils are also present in villi of the lamina propria harboring signs of immune stimulation with the presence of Peyer's patches. One difference between the tonsil and the small intestine is that epithelial cells do not produce APRIL in the latter. Hence, lymphoid organs can be divided in two, those with a constitutive production of APRIL, bone marrow and spleen, and those with an inflammation-induced production of APRIL, MALT. It is noteworthy that retention of secreted APRIL is also very different between the two. In bone marrow and spleen, there is almost no retention of secreted APRIL (manuscript in preparation), while secreted APRIL is retained and concentrated in specific areas of MALT. The absence of detectable secreted APRIL in the bone marrow is unexpected, since it plays an essential role for PC survival in this organ (Belnoue et al., 2008). The low level of secreted APRIL retained correlates with the paucity of HSPG staining in the human bone marrows we studied (unpublished observation). This observation is consistent with the fact that HSPG expression has never been reported in situ in human bone marrow, at least to our knowledge. However, this absence of staining contrasts with the fact that PC express constitutively CD138; an HSPG able to bind secreted APRIL. In situ, the proteic core of CD138 is observed at PC surface from bone marrow, but there is no detection of heparan sulfate side chains on this molecule. One possibility is that bone marrow PC consume heparan sulfates and the APRIL bound to them. A similar situation may well apply for the spleen, wherein short-lived PC are present (Kunkel and Butcher, 2003). In MALT, APRIL is efficiently retained, and the retention correlates well with PC homing. Indeed, APRIL retention is observed in the sub-epithelial zone of mucosa, exactly where PC home upon mucosa inflammation (Brandtzaeg and Johansen, 2005). The HSPG, CD138, also known as syndecan-1 (Huard et al., 2008), and to a lesser extent syndecan-4 (unpublished observation) from basal tonsil epithelial cells bind secreted APRIL. In the small intestine, there is a little retention on the basal surface of CD138-expressing epithelial cells, and sub-epithelial cells, whose identity are still undefined, in the vicinity of intestinal PC retain most of the product. In these two cases, APRIL is presented by HSPG in trans to PC. APRIL bound directly onto PC by HSPG can be detected in MALT, providing a cis presentation. However, the major fraction of PC is devoid of secreted APRIL on their surface, suggesting again consumption of the molecule by PC. Taken together, APRIL-rich niches are created upon inflammation in the mucosa. These niches provide a favorable environment for PC, thus mediating a local frontline humoral defense. In spleen and bone marrow, the APRIL supply for PC is constitutive.

APRIL and tumor development

APRIL was first identified as a tumor-promoting molecule, warranting the study of APRIL expression in tumors. Reactivity against stalk and secreted APRIL was observed in tumors, but striking variations in the level of APRIL expression were observed, even among patients with the same disease. Some patients had abundant cells producing APRIL, associated with a high retention of secreted APRIL by HSPG in their lesions, while others harbored very few, if any, APRIL-producing cells and secreted APRIL. In most cases, variation in APRIL expression did not correlate with standard clinical parameters, so that the pathway(s) responsible for such variations is presently not known. At the tumor-cell level, a stalk reactivity was not observed in malignant cells from Hodgkin lymphoma (HL) (n=285), non-Hodgkin lymphoma (NHL) (n=399), and in more than 90% (n=2158) of solid tumors, showing that autocrine production of APRIL by tumor cells is rare, at least when a level of expression detectable by immunohistochemistry is considered. In the few positive cases of solid tumors, the stalk reactivity was not homogeneous, but focal among the tumor nest, indicating that APRIL production by tumor cells is not constitutive, but induced under particular circumstances, perhaps similar to the inflammatory trigger that induces production in epithelial cells. Hence, while APRIL mRNA is widely observed in tumor cell lines, APRIL protein production in vivo is not frequently detected at

the tumor cell level. As for normal tissues, neutrophils infiltrating tumor lesions are the main cellular source of APRIL. Only a minority of NHL lesions harbor L protein-expressing histiocytes or vimentin-expressing mesenchymal cells producing APRIL. Similarly to healthy tissues, APRIL-producing cells secrete APRIL, and the secreted product is efficiently retained in tumor lesions, with a high concentration directly bound onto tumor cells, again by HSPG.

The variations observed in the level of APRIL expression in tumor lesions allowed patient stratification into high and low expressers, enabling studies on the role of APRIL in the clinical development of tumors. Tumor lesions of about half of the patients with various solid tumors from a large cohort (n=2158) showed APRIL upregulation ($APRIL^{high}$) (Mhawech-Fauceglia et al., 2006). Retrospective analysis of disease-free (DFS) and overall (OS) survival for patients with bladder, ovarian or head and neck carcinoma, stratified according to APRIL expression, revealed no significant differences between the $APRIL^{low}$ and $APRIL^{high}$ groups (Mhawech-Fauceglia et al., 2008). An independent investigation showed that more than 70% of breast cancer patients are APRILhigh (Pelekanou et al., 2008). Likewise, APRIL upregulation in these patients did not modulate DFS or OS. Hence, the early reports showing a promoting role for APRIL in the natural course of solid tumors are not confirmed in treated patients.

Some HL lesions, characterized by Reed-Sternberg tumor cells, also showed APRIL upregulation. Intralesional APRIL is upregulated in 64% (n=285) of these patients. Reed-Sternberg cells are often tightly associated with APRIL-producing neutrophils (Schwaller et al., 2007), and as a consequence are exposed to high concentrations of APRIL. Nevertheless, we found only trends towards lower DFS and OS in $APRIL^{high}$ patients that did not reach statistical significance (Went et al., 2008). It is notable that the impact on survival for a single tumor-promoting biological marker such as APRIL in HL-patients, may be masked by the high treatment efficacy currently achieved in this disease (Diehl et al., 2005). Indeed, 92% of patients in the cohort we analyzed were still alive after 15 years. Because APRIL upregulation did not modulate the occurrence of HL relapse, it is unlikely that APRIL will become a valuable biological marker for prognosis and/or treatment of HL.

APRIL upregulation is also observed in NHL. Seventy-five percent of chronic lymphocytic leukemia (CLL) patients (n=95) had higher than normal amounts of APRIL in their blood (Planelles et al., 2007). Notably, the OS of $APRIL^{high}$ patients was significantly lower, and the $APRIL^{high}$ parameter almost doubled the prognostic value of the clinical parameters currently used for CLL. This clinical study corroborates well the preclinical data obtained from APRIL transgenic mice that develop B-cell lymphoma reminiscent of human CLL (Planelles et al., 2004). Taken together, this indicates that APRIL may be a valuable biological marker for CLL prognosis and/or treatment. Accordingly, a soluble form of TACI used as a decoy receptor, Atacicept, was tested in a phase Ib study, during which one of the 12 patients with refractory and/or relapsed CLL enrolled patients showed disease stabilization (Koffler et al., 2007). Notably, solid lesions of CLL in lymphoid organs did not show APRIL upregulation (Schwaller et al., 2007), indicating that APRIL may promote the development of the leukemia but not the lymphoma form of CLL. In contrast, two other NHL, Burkitt lymphoma (BL) and diffuse large B-cell lymphoma (DLBCL) showed APRIL upregulation in tumor lesions (Schwaller et al., 2007). In DLBCL, APRIL was upregulated in 46% of lesions (n=56), and DFS and OS of $APRIL^{high}$ patients were significantly decreased. We have been able to confirm recently the decreased survival among $APRIL^{high}$ patients in a second independent cohort of 107 patients (unpublished observations). In this second cohort, a multivariate analysis demonstrated that the $APRIL^{high}$ parameter increases the value of the international prognostic index (IPI) currently used for DLBCL by more than two fold. APRIL acts most likely by increasing the resistance of tumor cells to chemotherapy, as He at al. observed *in vitro* (He et al., 2004). Atacicept was also tested in 6 patients with DLBCL, but no significant clinical effect was found (Ansell et al., 2008). Because APRIL expression is extremely variable among patients, it may be useful to enroll only $APRIL^{high}$ DLBCL patients in future clinical trials. APRIL was also monitored in the serum of DLBCL patients (Kim et al., 2008). Serum APRIL levels were increased in 50% of the patients (n=66), but only a trend towards a lower progression-free survival (PFS) and OS of $APRIL^{high}$ patients was found. Although several technical differences between the two studies may account for this discrepancy, differences in cohort demographies are an obvious explanation. Among others, all patients in the studies in which APRIL was quantified in tumor lesions had received chemotherapy only, while the patients in the study based on serum levels were treated with chemotherapy in combination with Rituximab (R-CHOP). It is conceivable that the effect of APRIL may be much less in patients treated with R-CHOP considered to be of higher potency. Hence, further studies are warranted for the role of in situ APRIL upregulation in the development of DLBCL from patients receiving R-CHOP. Taken together, these clinical studies indicate that APRIL may be a valuable biological marker for CLL and DLBCL. Prospective studies have to be performed to demonstrate whether the APRIL parameter might be used in risk assessment. If it is indeed the case, one may think of targeting APRIL for drug resistant CLL and DLBCL.

Concluding remarks

APRIL is a "good" molecule to study its expression in tissues. First, the stability of the stalk fragment in

producing cells allows detection of the latter. Second, the retention of the soluble product by HSPG allows a precise localization of secreted APRIL. Such tissue reactivity may well apply to other, similarly processed, TNF ligands. For APRIL, in situ expression led to valuable informations on its (patho) physiological roles. APRIL-rich niches are observed in an inflamed mucosa. In these niches, PC accumulate and survive during the time of the infection, hence providing a frontline humoral defense. In the spleen and bone marrow, the APRIL supply for PC is constitutive. In tumor lesions, APRIL is also retained, in some instances, with a very high level of secreted APRIL bound directly onto tumor cells, providing them with an important survival/ proliferation signal to support growth and treatment resistance. In all the tissues analyzed to date, HSPG play a key role in the process of APRIL retention, whose main cellular source are neutrophils.

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