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Apoptosis and autoimmune thyroid disease: following a TRAIL to thyroid destruction?

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In the past decade, it became apparent that immune mediated cell death in a number of autoimmune endocrine diseases was due to the induction of apoptosis in target organ cells. This was conclusively demonstrated for thyroid follicular cells in Hashimoto's (destructive autoimmune) thyroiditis, but the mechanisms underlying this cell death were not clear. Several hypotheses were put forth involving the role of death-signalling molecules expressed on thyroid cells. While many of these hypotheses did not hold up under close scrutiny, this stimulated work on the molecular mechanisms of thyroid destruction. Several apoptosis signalling pathways, initiated by molecules such as Fas ligand (FASL) and tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), have been shown to be active in thyroid cells and may be involved in destructive thyroiditis. In this review we will attempt to sort out the inconsistencies in published data on the mechanisms of death-receptor mediated thyroid destruction. We will also review recently proposed models of these mechanisms, and outline directions for research that we feel might lead to discoveries of benefit to the clinician in the treatment and prevention of destructive autoimmune thyroiditis.

Autoimmunity-induced hypothyroidism: an apoptosis-mediated disease

Hashimoto's thyroiditis is a disease characterised by an immune cell infiltrate, presence of thyroid-specific autoantibodies and thyroid autoantigen-specific T lymphocytes, and the destruction of follicular structure. While it is often diagnosed in a euthyroid patient on the basis of goitre and autoantibodies, over time this usually progresses to a deficit in thyroid hormone production and the symptoms of hypothyroidism. The most important feature for our discussion is the destructive nature of this disease and we will refer to this as 'destructive (autoimmune) thyroiditis' to differentiate it from the other thyroiditis syndromes that do not cause glandular destruction.

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The study of autoimmune thyroiditis has been plagued by the difficulties in examining a disease that progresses over long periods of time (Costa *et al*., 1989; Davies & Amino, 1993; Dayan & Daniels, 1996). The factors that start the autoimmune destruction are unknown because they occur long before the diagnosis is made. In addition, the destruction of thyroid cells occurs slowly, with cell death occurring over years. While there are other contributing factors such as TSH receptor blocking antibodies or cytokine inhibition of thyroid function, that may play a role in thyroid dysfunction, we will focus on the mechanisms of thyroid follicular cell elimination as the primary (Dayan & Daniels, 1996) cause of thyroid hormone deficit in this disease. This thyroid cell elimination is now known to occur through the process of apoptosis.

While Hashimoto described thyroid destruction in his initial observations in 1912 (Hashimoto, 1912), only recently has this cellular destruction been recognized as apoptotic (Kotani *et al*., 1995; Okayasu *et al*., 1995; Tanimoto *et al*., 1995). Apoptosis is a mechanism that allows cells to self-destruct when stimulated by the appropriate trigger. This process is initiated for various reasons, such as when a cell is no longer needed within the body or when it becomes a threat to the health of the organism. The aberrant inhibition or initiation of apoptosis contributes to many disease processes (Encyclopædia Britannica Online,. 2000). It has been calculated from experiments on animal thyroids and human thyroid organ cultures that the thyroid gland turns over approximately 5 times during a lifetime (Coclet *et al*., 1989). Cells must be continually eliminated to compensate for the production of new thyroid follicular cells in order to maintain normal thyroid size and function. Normal thyroid glands show a low level of apoptosis, suggesting a role for this process in basal thyroid cell turnover (Dremier *et al*., 1994; Kotani *et al*., 1995; Okayasu *et al*., 1995; Tanimoto *et al*., 1995). In contrast thyroid cells undergoing apoptosis (as determined by immunohistochemical and morphological analyses) occur with increased frequency in thyroids from patients with destructive thyroiditis (Kotani *et al*., 1995; Okayasu *et al*., 1995; Tanimoto *et al*., 1995). Many of the apoptotic cells in these glands are detected in areas of disrupted follicles in proximity to infiltrating lymphoid cells (Kotani *et al*., 1995; Hammond *et al*., 1997). This suggests that the thyroid destruction in this disease occurs through thyroid cell apoptosis.

An important point in evaluating the role apoptosis plays in

mediating destructive thyroiditis is how apoptosis is defined and detected. At this point it should be noted that some of the experiments identifying apoptosis in thyroid cells used *in situ* (TdT-mediated dUTP Nick-End labelling) TUNEL (ApopTag®) staining that detects DNA fragmentation through the specific enzymatic labelling of the 3′ hydroxyl (OH) group of DNA molecules. Unfortunately, this technique alone does not differentiate between apoptosis and necrosis. This method must be used in conjunction with observation of cell morphology changes characteristic of apoptosis, and these are sometimes lost in immunohistochemical staining techniques. The morphological changes will be described in more detail in the next section. New, more specific techniques have been developed which include immunohistochemical detection of protein epitopes that are unique to cells undergoing apoptosis like caspase-cleaved Cytokeratin 18 or activated Caspase 3. Thus, future studies examining this issue should be better able to measure the extent of apoptosis in the thyroid gland.

Apoptosis: mechanisms and results

Apoptosis is an active, energy dependent cellular process. This can occur through several different initiating processes including growth factor deprivation, the toxic actions of radiation and chemotherapy drugs, and through endogenous molecules that act as receptors for cell-bound or soluble 'death' ligands. These ligands include tumour necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL). While the activating events start different signal cascades, all of these communications feed into a final, common apoptosis pathway that leads to cell death (Strasser *et al*. 2000). The common pathway consists of sequential activation of proteases, specifically known as Caspases. Caspases are cysteine proteases (defined by having a cysteine residue at their active site) which cleave proteins at specific peptide sequences that contain an aspartate residue (Salvesen & Dixit, 1997). These caspases are themselves activated by specific proteolytic cleavage. This cleavage can be performed by other caspases, thus amplifying the signal cascade. The caspase cascade ultimately activates enzymes that progressively digest the cell and its genetic material, producing the classic morphological changes that occur during apoptosis such as chromatin condensation, cytoplasmic shrinkage and plasma membrane blebbing (Wyllie *et al*., 1980; Wyllie, 1980). Specific endonuclease cleavage of DNA occurs, and this can be differentiated biochemically from the random DNA cleavage that occurs in necrosis because the DNA is cleaved only once for every turn around chromatin. This leads to a 'ladder' effect with DNA fragments that are multiples of 180 bases (Wyllie, 1980). Cell destruction proceeds in such a way that it signals engulfment of the dying cell by surrounding cells, thus preventing the immune response that occurs with necrotic release of components of damaged cells (Fadok *et al*. 2000; Savill & Fadok, 2000). Growth factor deprivation, radiation and chemotherapeutic agents initiate apoptosis through the p53 apoptosis pathway signalled through Apaf-1 and Caspase 9. These events are either enhanced or inhibited by the Bcl-2 family of proteins that appear to work in co-ordinated pairs (Strasser *et al*. 2000). These p53 activation pathways are not likely to be involved in autoimmune thyroid apoptosis and will not be discussed here.

In contrast, the apoptosis initiation processes that have gained so much attention recently are the death receptor-induced pathways (summarised in Fig. 1). Death receptors are members of the TNF receptor family of transmembrane signalling proteins that contain a cytoplasmic protein polymerisation domain known as a 'death domain' (Baker & Reddy, 1998; Aravind *et al*., 1999). The death receptors bind to and are activated by specific death ligands. Table 1 summarises the known death ligand/receptor interactions.

Receptor-mediated apoptosis can be regulated by controlling expression of the individual components (usually at the level of transcription) as well as by endogenous protein inhibitors. Endogenous inhibitors of receptor-mediated apoptosis include 'decoy' receptors (e.g. DcR1, DcR2, DcR3) (Ashkenazi & Dixit, 1999) that compete with death receptors for binding of death ligands and inhibitors of death-induced signalling complex (DISC) formation or initiators of caspase activation (e.g. cellular FLICE-like inhibitory protein (cFLIP)) (Irmler *et al*., 1997). Caspase inhibitors (e.g. inhibitor of apotosis protein (IAP) protein family) (Deveraux & Reed, 1999), as well as inhibitory proteins with as yet undefined mechanisms (e.g. fas-associated phosphotase-1 (FAP-1) have also been described (Sato *et al*., 1995). Regulation of expression of these molecules appears important for modulating the induction of apoptosis and provides important clues in designing possible interventions of the cell death signal (Nicholson, 2000).

Immune-mediated apoptosis

The immune system uses apoptosis to mediate cell-mediated cytotoxicity of target cells that must be eliminated from an organism. This has been shown to be important in at least two specific circumstances where the host's survival is at stake: (1) elimination of infected (both virus and bacteria) cells and (2) elimination of neoplastically transformed cells. Another example of cell-mediated cytotoxicity that is medically important is allograft rejection. However, if the immune system eliminates a normal cell that is neither infected nor transformed (nor foreign), then it is described as autoimmune destruction and this may result in autoimmune disease. Cell-mediated cytotoxicity is initiated through two different mechanisms: (1) effector cell expression of death ligands presented to target cells and (2) effector cell release of exocytic

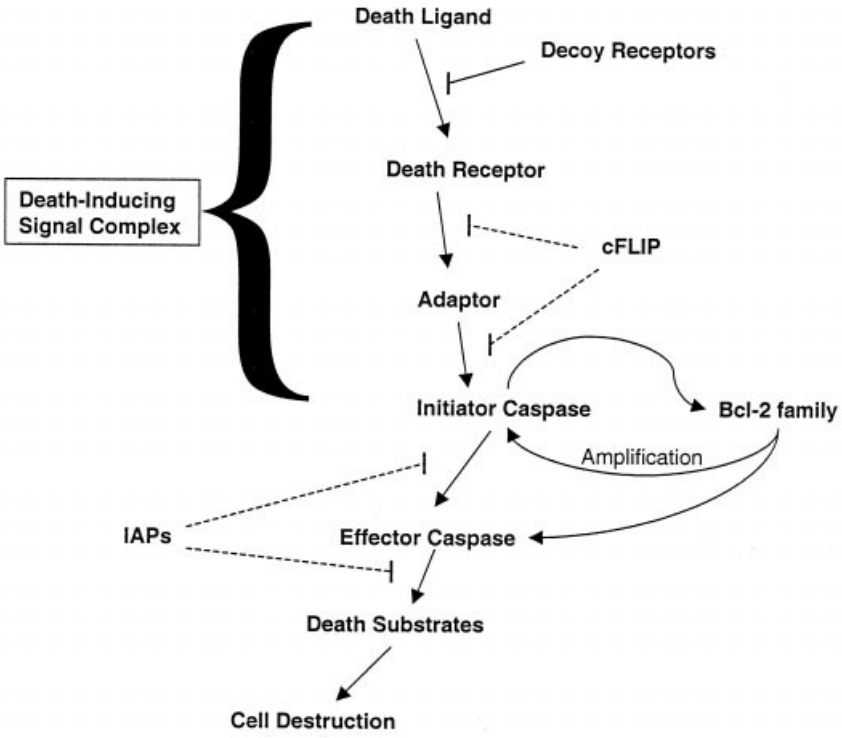

Fig. 1 Components of the death receptor-mediated apoptosis signalling pathway.

granules containing perforin (which perforates target cell membranes) and Granzyme B (which initiates apoptosis by directly activating caspases by proteolytic cleavage (Talanian *et al*., 1997; Van de Craen *et al*., 1997). The first case requires functional death receptor expression by the target cell. T lymphocytes and NK cells, both well characterised cytotoxic cells, have been shown to functionally express the death ligands TNFα, FasL, and TRAIL when they become activated (Mariani & Krammer, 1998; Zamai *et al*., 1998; Kayagaki *et al*., 1999).

As mentioned previously, apoptosis is involved in maintaining a proper balance in cell populations. Apoptosis eliminates cells in order to maintain a constant number of cells within a proliferating population, or to eliminate cells no longer needed. An example of this is following an immune response when expanded populations of lymphocytes responding to a perceived threat need to be restrained. Without reducing the number of these cells an ongoing inflammatory response could damage normal cells (known as the 'bystander effect'). To reduce the number of these cells, death ligands on some of the immune cells bind to, and specifically activate, the death receptors on other immune cells. The result is that the activated immune cells eliminate each other by either apoptotic suicide or fratricide. Loss of the ability to reduce immune cell numbers through this mechanism results in unregulated expansion (hyperplasia) of immune cells leading to lymphoproliferative autoimmunity. Examples of this include a Lupus-like disease of Fas and FasL deficient mice (Cohen & Eisenberg, 1992) and Autoimmune Lymphoproliferative Syndrome (ALPS) (Fisher

Table 1 Death receptor/ligand interactions and decoy receptors

Death receptor	Ligand	Decoy receptors
Fas (CD95)	FasL (CD95L)	DcR3, soluble Fas
TNF-RI	TNFα, Lymphotoxin	soluble TNF-R
Death receptor 3	Apo3L/TWEAK	–
Death receptor 4	TRAIL (Apo2L)	DcR1 (TRAIL-R3, TRID, LIT), DcR2(TRAIL-R4,TRUNDD)
Death receptor 5	TRAIL	DcR1, DcR2
Death receptor 6	unknown	–

et al., 1995; Dianzani *et al*., 1997). The former condition results in organ-specific autoimmunity, including thyroiditis and hypothyroidism, in addition to the Lupus-like syndrome (Green *et al*., 1995a; Green *et al*., 1995b). ALPS in humans has been linked to mutations in Fas, FasL and Caspase 10, but the autoimmune response is neither organ-specific nor is it apoptotically destructive of any specific organ. It is possible that thyroid specific autoimmunity in humans may be influenced by a similar generalised loss of immune control, but this has yet to be documented. Support for this statement comes from the fact that autoimmune thyroiditis is often associated with other autoimmune diseases such as diabetes and Addison's disease, possibly suggesting a common defect predisposing to apoptotic destruction of the endocrine organs (Baker, 1992).

Inflammatory cytokines have numerous effects on the immune system including the regulation of apoptosis. Cytokines such as interferon γ (IFNγ), interleukin-1 (IL-1) and TNF can influence immune-mediated apoptosis both directly and indirectly. Cytokines can regulate expression levels of apoptosis pathway components and inhibitors in target cells, as well as regulate effector cell expression of apoptosis initiators. They can also promote apoptotic activity in effector cells by promoting growth, migration and activation of these cells.

Many inflammatory cytokines are present in an inflamed thyroid. Experimental evidence for a role of these cytokines in autoimmune thyroid disease has been well-documented (Kawakami *et al*., 1990; Frohman *et al*., 1991; Stull *et al*., 1992; Tang *et al*., 1993; Ajjan *et al*., 1996; Schuppert *et al*., 1996; Alimi *et al*., 1998). A role for inflammatory cytokines in the effector phase of destructive thyroiditis may lie in the regulation of thyroid follicular cell susceptibility to immune-directed, death receptor-mediated apoptosis. This information combined with the previously mentioned observation of infiltrating immune cells adjacent to apoptotic thyroid follicular cells strongly supports a role for the participation of immune-mediated apoptosis in the effector phase of destructive thyroiditis. The molecular mechanisms for mediating this apoptotic death in the thyroid are currently being elucidated.

Receptor-mediated apoptosis in thyroid cells

A variety of different models have been proposed to describe immune cell destruction of thyroid cells. Each of the models assumes that some sort of an immune response to thyroid autoantigens occurs and stimulates an immune cell infiltration into the thyroid gland (initiator phase), and this then leads to thyroid cell death (effector phase). However, the existence of inflammatory cell infiltrates in the thyroid does not inevitably lead to thyroid destruction. There are a number of thyroiditis syndromes that occur without gland destruction (Davies & Amino, 1993). The change from non-destructive thyroiditis to destructive thyroiditis occurs by an unknown mechanism. This suggests that the thyroid cells may play a role in this process (Volpe, 1994). Because of this it is most important to identify the molecular signalling of apoptotic destruction in the thyroid gland during thyroiditis.

In 1997, Giordano and colleagues suggested that thyroid cells were killing themselves by coexpressing Fas and FasL under conditions found in an inflamed thyroid (Giordano *et al*., 1997). Their hypothesis was based on data that showed constitutive expression of FasL on thyroid follicular cells from goitre and thyroiditis tissue (Giordano *et al*., 1997), while Fas appeared to be expressed only in thyroids from destructive thyroiditis patients. These investigators also showed data suggesting that IL-1β, an inflammatory cytokine found in abundance in thyroiditis, could induce functional expression of Fas in thyroid cells in culture (Giordano *et al*., 1997). They concluded that the constitutively expressed FasL in the thyroid could induce apoptosis in thyroid cells that are exposed to IL-1β provided by the infiltrating immune cells. However, this intriguing hypothesis (Williams, 1997) was inadvertently based on data that had major technical flaws. In a rare convergence of bad fortune, the investigators used two commercial antibodies marketed as being specific for FasL. However, these antibodies were later proven to give false positive results in western blot analysis (Fiedler *et al*., 1998; Baker & Bretz, 2000; Fiedler & Eibel, 2000), flow cytometry (Smith *et al*., 1998) and immunohistochemistry (Strater *et al*. 2000). In addition, they used a reverse transcriptase polymerase chain reaction (RT-PCR) analysis of Fas and FasL that appeared to be flawed (Stokes *et al*., 1998) and used goiterous cells as a 'normal control' (Stokes *et al*., 1998). In a follow-up technical commentary, the authors demonstrated that other FasL specific antibodies also detected FasL in thyroid cells. However, these antibodies also have been shown to be inadequate for FasL detection (Strater *et al*. 2000)[1]. Immunohistochemical staining of normal thyroid was presented which

[1] Strater *et al*. (2000) have demonstrated that of 12 commercially available antibodies tested for immunohistochemical staining, 3 showed false positive results (clone 33, C-20, N-20; overestimated FasL expression), 8 showed false negative results (NOK-1, NOK-2, 4H9, MIKE-1, MIKE-2, 8B8, A11, 4A5) and only one (G247-4 from Pharmingen) accurately reflected expression as demonstrated by *in situ* hybridization detection of FasL mRNA. Although Strater *et al*. (2000) Temp. only address the specificity of these antibodies in immunohistochemical staining, this data combined with the previously published problems concerning antibodies used for FasL detection by other methods, make it critical that FasL expression be carefully evaluated using multiple antibodies that have demonstrated reliability that is confirmed by mRNA detection techniques.

the authors described as FasL, however, these follicular cells have atypical thyroid follicle morphology (Papoff *et al*., 1998). Others have used different antibodies to demonstrate FasL expression in the thyroid in Graves' disease (Hiromatsu *et al*., 1999b) and papillary thyroid cancer (Mitsiades *et al*. 2000a), but their results are also confounded by the use of antibodies that either give false positive or false negative results (Strater *et al*. 2000)[1]. The antibodies that specifically detect FasL expression *did* demonstrate expression in the thyroid (Hiromatsu *et al*., 1999b; Mitsiades *et al*. 2000a) but the relative levels of expression are difficult to evaluate due to the antibody's unreliability (Strater *et al*. 2000). In fact Mirakian *et al*. (1998), in a non-peer-reviewed commentary, stated that their group found that contrary to prior reports Hashimoto's thyroiditis-derived cells expressed less FasL than Graves' thyroid follicles, but the antibody they employed for this analysis was not reported. Inducible Fas expression in thyroid cells has recently been confirmed by another group (Kawakami *et al*., 1996), but this data was contradicted by the majority of publications which showed constitutive Fas expression by normal thyroid cells (Tanimoto *et al*., 1995; Arscott *et al*., 1997; Hammond *et al*., 1997). In summary, FasL expression occurs in the thyroid under some pathogenic conditions, but the correlation between expression and disease pathogenesis remains to be demonstrated.

In trying to examine function of the Fas pathway in thyroid cells, several unique observations have been made. Functional Fas expression has been reported in normal thyroid cells (Arscott *et al*., 1997; Bretz *et al*., 1999b), papillary thyroid cancer cells (Arscott *et al*., 1999), and thyroid cancer cell lines (Mitsiades *et al*. 2000a). However, function was only demonstrated when the cells were treated with the protein synthesis inhibitor cycloheximide or with inflammatory cytokines (Kawakami *et al*., 1996; Arscott *et al*., 1997; Bretz *et al*., 1999; Paolieri *et al*., 1999). This demonstrates that although Fas protein is expressed on thyroid cells the pathway is regulated and inhibited under normal conditions. The optimal condition for the induction of Fas-mediated apoptosis in thyroid cells requires pretreatment with IFNγ and TNFα (Bretz *et al*., 1999b). Whereas untreated cells are totally resistant to FasL, apoptosis is detectable in cytokine pretreated cells within 2 h and is nearly completed within 7 h of Fas-activation (Bretz *et al*., 1999b). Reports on Fas-mediated thyroid cell death under other conditions required significantly longer, non-physiologic time periods (Kawakami *et al*., 1996; Giordano *et al*., 1997; Kawakami *et al*., 1997), calling into question whether the death was truly the result of Fas-mediated apoptosis.

Additional recent publications also seem to argue against Fas-mediated thyroid suicide (as proposed by Giordano *et al.,* 1997). Mice genetically manipulated to specifically over-express FasL in the thyroid do not have increased apoptosis in thyroid cells (Batteux *et al*. 1999, Batteux *et al*. 2000). When immune cell infiltrates are induced in these mice by injection with thyroid autoantigen, there was a reduction in inflammation in mice expressing the transgene as compared to control animals (Batteux *et al*. 2000; Batteux *et al*., 1999b). Mitsiades *et al*. 2000b recently described evidence that the thyroid inhibitor drug methimazole can induce FasL expression in thyroid cells. They propose the hypothesis that the methimazole-induced FasL contributes to the immunomodulatory effect of this drug by killing infiltrating lymphocytes, but not thyroid cells where Fas pathway function is inhibited. This data also argues against the theory that FasL is constitutively expressed on thyrocytes. Soluble Fas expression has been reported in Graves' disease, and is even observable in the serum of individuals with this disorder (Shimaoka *et al*., 1998; Hiromatsu *et al*., 1999a). There does not appear to be toxic effect from this level of soluble FasL as there is no evidence of hepatocyte apoptosis in these patients. However, a potential role for soluble FasL could exist in Graves' disease given the reports of complications, such as liver and cardiac dysfunction, that occur in this disorder. In summary, it is unlikely that thyroid cells kill themselves through self-expressed FasL/Fas interaction. While FasL or Fas most likely both exist on the thyroid and are functional under unique conditions, no experimental data proving a causal relationship between Fas and autoimmune thyroid disease has been produced. In contrast, there appears to be some data supporting anti-inflammatory and immune surveillance roles for the Fas pathway in thyroid disease. Given the data produced by Giordano *et al*. (1997) Dayan and colleagues proposed that the FasL expression by thyrocytes could be combined with their ability to express MHC class II proteins after exposure to cytokines (Dayan *et al*. 1997). This would allow the thyrocytes to eliminate Fas-expressing thyroid antigen-specific (autoreactive) T lymphocytes that would bind the thyroid cell expressed MHC class II antigens through the T cell receptor. This is bolstered by evidence that shows inflammatory cytokines can also upregulate cell adhesion molecules like ICAM and LFA that can tighten the cell-to-cell contact thus increasing interactions that could lead to induction of apoptosis (Paolieri *et al*., 1999). Several groups have shown thyroid cells functionally capable of killing Fas-susceptible cells (Giordano *et al*., 1997; Hiromatsu *et al*., 1999b; Mitsiades *et al*., 1999; Mitsiades *et al*. 2000b) and Fas expressing lymphocytes are present in thyroid infiltrates (Hammond *et al*., 1997; Stassi *et al*., 1999). A report documenting the reduction of immune infiltration by transgenic expression of thyroid cell FasL in the mouse experimental autoimmune thyroiditis (EAT) model supports this concept (Batteux *et al*. 2000). A defect in the ability to fight off immune cells would lead to thyroid destruction.

The conventional model of immune-mediated thyroid destruction asserts that antigen-specific cytotoxic immune cells destroy thyroid cells. Thyroid-derived and peripheral blood lymphocytes, when activated, have been shown to be cytotoxic to thyroid cells (Creemers *et al*., 1983; Sack *et al*., 1986; MacKenzie *et al*., 1987; Kawakami *et al*. 2000). However, this activity may be a normal function for eliminating neoplastically transformed cells known as 'immune surveillance'. The presence of FasL expressing lymphocytes in destructive thyroiditis also supports this model (Mitsiades *et al*., 1998). Several groups have argued against this model because they found very low levels of FasL expression by infiltrating cells (Mitsiades *et al*., 1998; Stassi *et al*., 1999). Again, this data was complicated by the use of antibodies that underestimate FasL expression by immunohistochemical staining (Strater *et al*. 2000)[1]. Flow cytometry data in one of these papers showed 12% of the infiltrating T lymphocytes were FasL positive which is arguably a significant percentage for inducing apoptosis in thyroid cells (Stassi *et al*., 1999). One group has published in a non-peer-reviewed article that a proteinase commonly used in tissue preparation can inhibit FasL activity (Palazzo *et al*. 2000). Preparation of tissues with this proteinase may digest FasL and cause underestimation of its expression. This may account for the low expression levels in thyroid infiltrating lymphocytes as determined by flow cytometry. It would appear that the mechanisms are in place for thyroid cells and thyroid-infiltrating immune cells to either 'kill or be killed'. The results would be destructive autoimmunity in one extreme or progression of a thyroid neoplasm in the other. It may be the careful balance between these outcomes that is required for the health of the thyroid. We can conclude from the data cited above and below that the combinations of inflammatory cytokines present in the thyroid microenvironment have an important role in this balance. We have reviewed this in more detail previously (Bretz & Baker, 2000).

The 'TRAIL' leading to thyroid destruction

We have recently generated data to support an alternative or perhaps complimentary model of death-receptor-mediated apoptotic destruction of thyroid follicular cells. TRAIL is a death ligand with significant homology to FasL. TRAIL acts through its receptors, Death Receptor 4 and Death Receptor 5, to induce apoptosis. We have demonstrated expression of these death receptors on thyroid follicular cells by RNase protection assay (Bretz *et al*., 1999a), western analysis and immunohistochemical staining (Bretz *et al*., unpublished data), each by two different antibodies. We also showed regulation of these receptors by inflammatory cytokines and increased expression in destructive thyroiditis (Bretz *et al*, unpublished data). Recombinant TRAIL is capable of killing thyroid cells (presumably through Death Receptor 4 and/or Death Receptor 5) only after cycloheximide treatment suggesting the existence of a labile inhibitor. Pretreatment with the unique combination of TNFα and IL-1β, demonstrating that the inhibitor is not the same as that postulated for the Fas pathway (Bretz *et al*., 1999b; Bretz *et al*., unpublished data). Interestingly, we have also demonstrated inflammatory cytokine induced functional expression of TRAIL by thyroid cells (Bretz *et al*., 1999a). Expression of TRAIL is strongest after treatment of the cells with IFNγ in combination with TNFα or IL-1β. Intriguingly, these are the best conditions for Fas-mediated death of these cells (Bretz *et al*., 1999b), but they are different from the conditions for TRAIL-induced killing (Bretz *et al*., unpublished data). Cells expressing TRAIL were capable of killing a TRAIL-susceptible lymphoid cell line and a TRAIL-neutralising antibody blocked this activity (Bretz *et al*., unpublished data). Immunohistochemical staining of normal thyroid tissue also showed TRAIL expression and this staining was increased in thyroid follicles undergoing destruction in tissue derived from patients with destructive thyroiditis (Bretz *et al*., unpublished data). TRAIL is known to be expressed by activated lymphocytes and capable of effecting cell-mediated cytotoxicity. We also showed that TRAIL mRNA is expressed in thyroid-infiltrating lymphocytes (Bretz *et al*., 1999a).

The information above can be applied to constructing models for the involvement of TRAIL in destructive thyroiditis similar to the FasL models. These models have been outlined previously (Bretz *et al*., 1999a; Bretz & Baker, 2000) and are summarised schematically in Fig. 2. In brief, the above mentioned data supports the hypotheses that TRAIL may be involved in thyroid follicle destruction by immune cells expressing TRAIL, or alternatively TRAIL may be involved in a defence mechanism for the thyroid cells to resist immune cell attack. In support of the later model it has been shown that TRAIL death receptors are expressed on autoreactive immune cells (Wendling *et al*. 2000). Also, it is possible that inflammatory cytokine induced TRAIL, when it interacts with cytokine activated TRAIL death receptors on the same thyroid cell, would lead to cell suicide or fratricide. This scenario is unlikely due to the fact that different cytokine combinations are required for these two activities (TRAIL induction by IFNγ/TNFα and TRAIL susceptibility by TNFα/IL-1β). The combination of all three cytokines inhibits TRAIL-induced killing (Bretz *et al*., unpublished data). It is possible that a defect in these signals could lead to thyroid cell suicide. The switch that regulates whether TRAIL expression results in killing of thyroid cells or intrathyroidal lymphocytes is probably the presence of IFNγ. IFNγ protects thyroid cells from TRAIL-mediated apoptosis and also provides a key signal for the induction of TRAIL by those

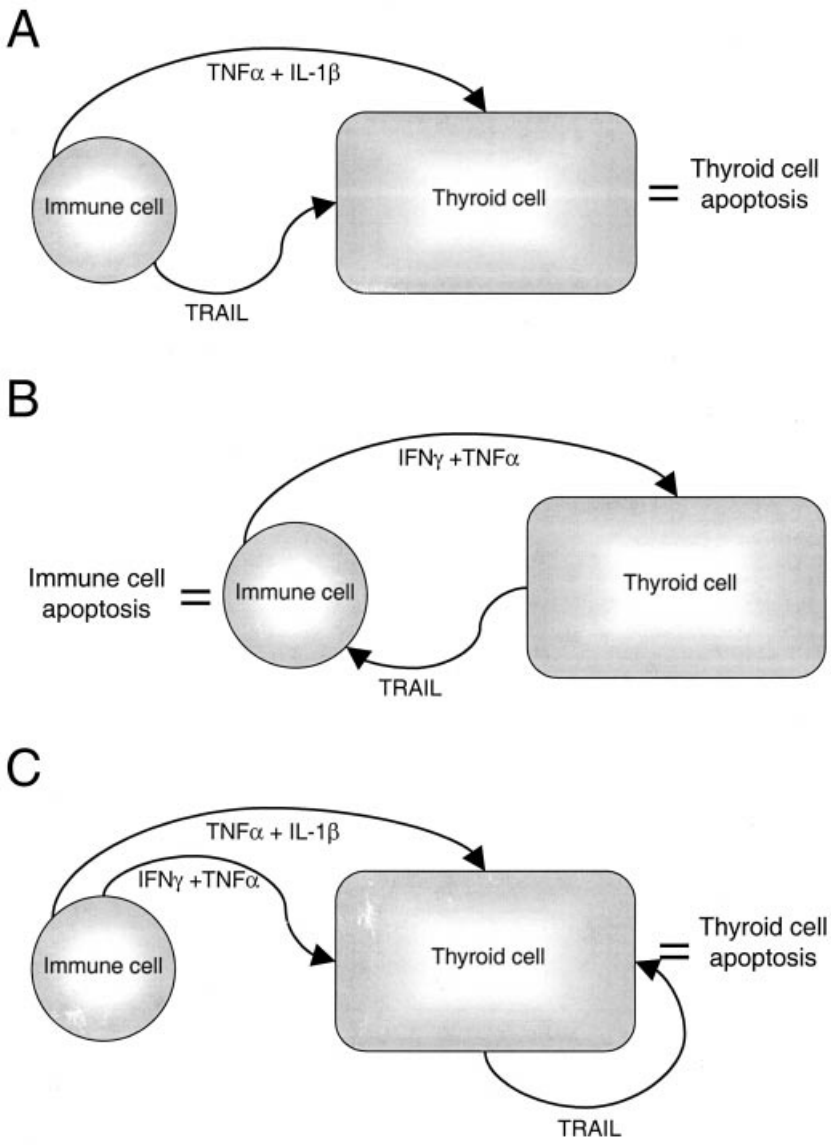

Fig. 2 Potential models for the involvement of TRAIL in autoimmune destructive thyroiditis. (a) The combination of inflammatory cytokines TNFα and IL-1β, produced by activated immune cells, induce thyroid cell susceptibility to TRAIL. Immune cells expressing TRAIL can subsequently kill the thyroid cells. Killing would be restricted to thyroid cells by activation of thyroid specific T lymphocytes that were presented thyroid autoantigens. (b) The combination of inflammatory cytokines IFNγ and TNFα, produced by activated immune cells, induce TRAIL expression by thyroid cells. Immune cells that have become activated by thyroid autoantigen presentation are susceptible to TRAIL and only thyroid autoantigen-specific immune cells will be killed. If the thyroid cells are cancerous then the tumour may use this mechanism to defend against immune attack, a process known as 'immune evasion'. (c) Cytokine induction of both thyroid expression of TRAIL (by IFNγ combined with TNFα) and thyroid susceptibility to TRAIL (by TNFα combined with IL-1β) would lead to the thyroid cell killing itself.

same thyroid cells. IFNγ also plays a role in activation of T lymphocytes which can result in their susceptibility to TRAIL. In the absence of IFNγ the thyroid cells may become susceptible to TRAIL and do not express any TRAIL for their defence. As previously mentioned, it is noteworthy that IFNγ also appears to be a key ingredient in providing a signal that makes thyroid cells susceptible to FasL-induced apoptosis.

There is also evidence to support the notion that TRAIL is more likely than FasL to be involved in thyroid gland destruction. TRAIL killing is specific for thyroid epithelial cells (Bretz *et al*., unpublished data) while Fas-mediated killing also occurs in thyroid-derived fibroblasts and vascular smooth muscle cells treated with the same inflammatory cytokine combinations that promote thyroid cell death (Bretz, unpublished data). Apoptosis of fibroblasts or vascular smooth muscle cells has not been reported in thyroiditis. In fact fibrosis is common in destructive thyroiditis. It is obvious that more data is needed to definitively support any of these models. The pattern of cytokine regulation of TRAIL expression by, and specific TRAIL-induced killing of thyroid cells provides a more plausible model for its role in destructive thyroiditis than the Fas pathway. Although this data does not exclude a role for apoptosis induction through other death receptors in destructive thyroiditis, we believe that the data supports the investigation of TRAIL receptor-mediated

apoptosis as deserving an equal consideration for further study in this area of research.

Lack of evidence of a role for Bcl-2 in destructive thyroiditis

Bcl-2 is the prototype member of a family of protein regulators of apoptosis signalling (Reed, 1997; Adams & Cory, 1998; Green & Reed, 1998; Newton & Strasser, 1998; Strasser *et al*. 2000). It has been proposed that regulation of Bcl-2 or members of the Bcl-2 family may be involved in destructive thyroiditis (Palazzo *et al*. 2000). This hypothesis is based on the observation that Bcl-2 expression, as detected by immunohistochemical staining, is decreased in thyroid follicular cells in destructive thyroiditis when compared to follicles from normal thyroid tissue (Hammond *et al*., 1997; Mitsiades *et al*., 1998). Also, in non-peer-reviewed data, one group has argued that Bcl-x, a Bcl-2 homologue, is regulated by inflammatory cytokines in thyroid cells (Palazzo *et al*. 2000). The only mechanistic data to support this was the finding that antisense nucleic acid inhibition of Bcl-2 in a thyroid cancer cell line could enhance Fas-mediated apoptosis (Fujieda *et al*., 1998).

A careful review of the literature does not support this hypothesis. Other groups have not found the same correlation between reduced Bcl-2 expression and destructive thyroiditis (Okayasu *et al*., 1995) (Branet *et al*., 1996). But regardless of this observation it is unlikely that Bcl-2 plays a significant role in destructive thyroiditis for several reasons. Kawakami *et al*. (1996) showed no change in Bcl-2 expression in cultured normal thyroid cells that became susceptible to Fas-mediated apoptosis after inflammatory cytokine treatment. Elimination of Bcl-2 would be expected to result in spontaneous thyroid cell destruction but there have been no reports of thyroid abnormalities in Bcl-2 gene knockout mice (or in transgenic mice over expressing Bcl-2). The most compelling argument against a role for Bcl-2 in destructive thyroiditis is that recent findings suggest that Bcl-2, and other Bcl-2 family members, are not directly involved in regulating the apoptotic signal by death receptors prior to the point of signal irreversibility, but are only involved in regulating the amplification of the death signal (Strasser *et al*., 1995; Keogh *et al*. 2000; Strasser *et al*. 2000). Bcl-2 does not block death receptor signals; it only slows down the signal by inhibiting the amplification of that signal. Yet Bcl-2 is capable of inhibiting apoptosis initiated by other mechanisms such as stress signals like DNA damage, growth factor deprivation and treatment with corticosteroids (Strasser *et al*. 2000). These are unlikely to play a role in the effector phase of destructive autoimmune thyroiditis.

To further clarify this issue, several experiments in animal models should be employed. Over-expression of Bcl-2 could be accomplished in primary thyroid cells in culture or in transgenic mice and the Bcl-2 gene could be knocked out in mice specifically in the thyroid. These tools should be used to determine whether there is a role for Bcl-2 in death receptor-mediated apoptosis of these cells.

What's next? Outline for the future.

The evidence that we have reviewed is strongly suggestive of a role for death receptor-mediated apoptosis in destructive autoimmune thyroiditis. But the data has largely been produced *in vitro* or *in situ*, and is mostly just correlative. For this information to be translated into information to be used in clinical practice more definitive proof is required to first determine the precise mechanisms of thyroid apoptosis *in vivo*, and second to link this activity to destructive thyroiditis. To achieve this aim transgenic, gene knockout or Cre/LoxP (organ-specific targeted gene knockout) mice might be used in mouse thyroiditis models to determine the specific role of genes implicated in thyroid cell apoptosis (FasL, Fas, TRAIL, Death Receptor 4, Death Receptor 5, decoy receptors).

It has been postulated that a number of genes may contribute to an individual's susceptibility to autoimmune thyroiditis and that it is the total accumulation of some (but not necessarily all or any specific combination) of these genetic alterations combined with environmental factors that lead to disease. Although genetic defects in apoptosis-related pathways could produce a generalized problem, it is likely that thyroid-specific autoimmunity is initiated by genetic and environmental factors that cause thyroiditis, and apoptotic defects would then facilitate thyroid destruction. This concept is bolstered by the fact that many forms of thyroiditis do not result in thyroid destruction. Also, in animal models of autoimmunity, inflammatory phases can be mechanistically separated from target organ destruction (Kolb, 1997; Pilstrom *et al*., 1997; Dilts & Lafferty, 1999; Ohsako & Elkon, 1999; Pakala *et al*., 1999). Analysis attempting to link specific apoptotic genes to the development of destructive thyroiditis may be useful in understanding pathogenesis of these disorders. In this regard, several apoptosis signalling genes have been mapped to chromosomal regions genetically linked to autoimmune thyroid disease (Tomer *et al*., 1999). More precise genetic mapping, using the tools provided by the Human Genome Project, should clarify potential roles for these genes and may facilitate identification of genetically susceptible individuals for preventative therapies.

The differential regulation of the FasL and TRAIL pathways by inflammatory cytokines suggests the various apoptosis signalling mechanisms are controlled by distinct molecular mechanisms. We have postulated the existence of a labile inhibitor for each of these pathways (Arscott *et al*., 1997; Bretz *et al*., 1999a). It will be important to identify these inhibitors

and other regulatory molecules. This information might make it possible to design specific pharmaceutical interventions to restrain autoimmune thyroid destruction. Several agents that regulate apoptosis *in vitro* are currently being tested in preclinical and clinical trials and could potentially be available to the clinician in a few years (Nicholson, 2000). Hopefully, this will allow the interesting information developed on apoptosis to be translated into medical practice.

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