THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INFLAMMATORY CYTOKINE REGULATION OF DEATH RECEPTOR-MEDIATED APOPTOSIS IN THYROID EPITHELIAL CELLS

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INTRODUCTION

Apoptosis is a normal, active, genetically controlled process of cell death that does not require the participation of inflammatory processes. Apoptosis mediates several normal functions in human biology including elimination of unneeded or unwanted cells in development, organ homeostasis, immune regulation and immune defense. Aberrant apoptosis is involved in the pathogenesis of many human diseases: abnormal cell death results in excessive parenchymal cell loss while decreased cell death contributes to the development of hyperplasias and neoplasias.

The two most frequently investigated apoptosis signaling pathways with relevance to thyroid homeostasis are the FasL (Fas Ligand) and the TRAIL (Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand) pathways. FasL and TRAIL are members of the tumor necrosis factor family and act through type I membrane proteins called death receptors. FasL is expressed in activated T lymphocytes and in cells of immune-privileged organs. It participates in cell-mediated cytotoxicity and maintenance of immune homeostasis by eliminating activated immune cells at the end of inflammatory reactions. In contrast to FasL, TRAIL is expressed in a wide variety of normal tissues, suggesting that this pathway is highly regulated and protective mechanisms exist in normal cells. While normal cells are resistant to TRAIL, it has been reported to selectively kill the majority of cancer cells. TRAIL induces apoptosis by interacting with either of two death receptors, DR4 and DR5. Two additional "decoy" receptors for TRAIL, that cannot transmit an apoptotic signal but can competitively block signal transduction, have also been identified: DcR1 and DcR2.

Most recently, the important role of proteasome was established in the regulation of programmed cell death. The proteasome is an ATP-dependent multisubunit proteolytic complex, responsible for the degradation of most intracellular proteins. Proteasome inhibitors have been used to induce apoptosis in various cell types.

Clinical disease in Hashimoto's thyroiditis (HT) is caused by the specific cytotoxic destruction of thyroid epithelial cells (TECs) by infiltrating lymphocytes. Fas-mediated apoptosis has been proposed as the mechanism of thyroid cell destruction in HT. Other death receptors, such as DR4 and DR5 may also be involved in this process, but due to their very recent characterization have not been evaluated. Activated T lymphocytes expressing death ligands such as FasL and TRAIL may be responsible for inducing thyroid cell death during the immune response to thyroid autoantigens.

Inflammatory cytokines are involved in the regulation of apoptosis influencing the expression of apoptotic signaling components and inhibitors in target cells, as well as controlling the expression of apoptotic initiators in effector cells. Many inflammatory cytokines are present in the thyroid gland in autoimmune thyroid diseases. Normal primary thyrocytes are resistant to death receptor-mediated apoptosis. However, the combination of IFNγ and TNFα or IL-1β sensitizes thyroid cells to Fas-mediated cell death. The effect of cytokine pretreatment on the susceptibility of TECs to TRAIL has not been investigated. It was reported that TRAIL itself is expressed by the thyrocytes treated with proinflammatory cytokines. Regulation of death receptor pathways in the thyroid may be a potential mechanism in which inflammatory cytokines might act to promote the progression of HT.

Thyroiditis can be experimentally induced in mice bearing the H-2K haplotype by immunization with thyroglobulin (Tg) and adjuvants. In this experimental autoimmune thyroiditis (EAT) model, mice develop autoimmune responses characterized by the occurrence of circulating anti-Tg antibodies and infiltration of the thyroid gland by lymphoid cells, including $CD4^+$ and $CD8^+$ T cells. EAT mimics some of the immunologic manifestations of HT but at variance with the human disease it regresses spontaneously after several weeks without thyroid follicular disruption, and is not accompanied by signs of hypothyroidism. Several publications have suggested a crucial role for cytokines in the pathogenesis of EAT but the exact function of these molecules is not clear. There has also been no information on the *in vivo* effects of IFNγ and TNFα on the thyroid.

Nodular goiter is one of the most common endocrine diseases. The pathogenesis of nodule formation has been intensively studied and recently activating mutations in the TSH receptor and Gsα genes were identified in the development of toxic adenomas. However, the majority of non-hyperthyroid nodules do not demonstrate protooncogene mutations and the primary events in the pathogenesis of these nodular goiters are still unknown. The most widely accepted hypothesis of nodule formation argues that there is heterogeneity in the growth potential and function of individual thyrocytes. Increasing evidence suggest a role for growth factor production in the thyroid, leading to TSH-independent growth of thyroid nodules. A lack of growth inhibition may also participate in the tissue imbalance that results in nodular goiter. Normal thyroids show a low level of apoptosis, a possible result of basal thyroid cell turnover. In multinodular goiters similarly low apoptosis rates have been detected by immunohistochemistry. No studies have investigated the regulation of death receptor-mediated apoptosis in nodular goiter.

The limitations of animal models in cancer research are well known. Virus or chemically induced, highly antigenic experimental tumors in animals are irrelevant to spontaneously developing human tumors which are frequently nonimmunogenic. While *in vitro* systems provide a wealth of information about cellular and molecular biology of tumor cells, they are inadequate to study the complexity of human neoplastic diseases, the metastatic features, the experimental therapeutics and anti-tumor immunity. The immunodeficient mice provided unique possibility to investigate transplanted human cancers *in vivo*. Severe combined immunodeficient (SCID) mice are carrying an inherited defect of the recombinase system for antigen receptor genes. Therefore, SCID mice accept both human solid tissues and lymphocytes. The multitude of human cancers (lymphoma, leukaemia, lung, breast, ovarian, colon cancer, retinoblastoma, osteosarcoma, melanoma) was successfully transplanted in SCID mice. The implantation of human immune cells results in the repopulation of the mouse bone marrow and lymphoid organs by human lymphocytes. The process is called immune 'reconstitution', referring to the largely uncharacterized presence of the human immune system in these mice.

Differentiated thyroid cancer (DTC) is present in situ in 10% of all autopsy specimens, indicating that the preclinical form of this disease is very common. Lymphocytic infiltration is frequently observed in and around the tumor. In thyroid cancer patients with thyroiditis a better survival rate was observed. Regulation of tumor cell apoptosis is a promising way to improve anti-cancer therapy. The presence of death ligands on tumor infiltrating lymphocytes and the contribution of death receptor-mediated apoptosis to tumor cell killing in DTC is unknown. TRAIL was found effectively kill carcinoma cell lines originating from the follicular thyroid epithelium. However, as a reliable animal model of this disease was not available, no studies on primary thyroid tumors have been done regarding the induction of apoptosis or other forms of immunotherapy.

SPECIFIC AIMS

In the present studies we sought to examine

1. the inflammatory cytokine regulation of TRAIL-mediated apoptosis in human thyroid epithelial cells *in vitro*
2. the mechanism of IL-1β/TNFα-induced sensitization of thyroid cells to TRAIL
3. the expression of TRAIL and its receptors in the normal thyroid gland and in thyroid tissue from Hashimoto's thyroiditis
4. the effect of IFNγ/TNFα on the Fas-mediated apoptosis and thyroid destruction in a murine model of experimental autoimmune thyroiditis
5. the sensitivity of primary thyroid cells from goiter nodules to TRAIL- and Fas-mediated apoptosis
6. the influence of proinflammatory cytokines on goiter cell apoptosis
7. the regulation of TRAIL pathway in goiter cells
8. the proteasome activity in primary thyroid cells derived from normal thyroids, multinodular goiters and papillary cancers
9. the usefulness of the hu-PBL-SCID mouse model for the investigation of human thyroid tumors and interactions between the immune system and the tumor cells.

MATERIALS AND METHODS

Cell culture: Thyroid cells obtained at thyroidectomy from normal thyroids, multinodular goiters and papillary cancers were cultured in CellGro Complete media supplemented with 20% NuSerum IV and 10 mIU/ml bovine TSH.

Cytokines, TRAIL, agonist antibodies, soluble receptors and proteasome inhibitors: Cells were treated for four days with 100 U/ml IFNγ, 50 ng/ml TNFα or 50 U/ml IL-1β and then exposed overnight to 800 ng/ml TRAIL, 0.1-0.5 μg/ml agonist anti-DR5 or 1 μg/ml agonist anti-Fas antibodies. Soluble human DR5/IgG Fc chimera and human TNF-R1/IgG Fc chimera were used at 0.2 μg/ml concentration. Proteasome inhibitors, lactacystin and MG132 were added at 1 μM and 10 μM concentrations, respectively.

Cell viability was determined by fluorescein diacetate/propidium iodide staining, caspase-specific proteolytic cleavage of cytokeratin 18 (M30 CytoDEATH® monoclonal Ab) and Annexin V binding (Annexin V-FLUOS staining kit) and was quantitated by flow cytometry.

RNase protection assay was accomplished for the detection and quantitation of TRAIL receptors' and IAPs' (Inhibitor of Apoptosis Proteins) mRNA expression.

The protein expression of DR5, DR4, DcR2, DcR1, cFLIP and bcl-2 from cell lysates was determined by immunoblot analysis.

The purity of thyroid cell population was checked by anti-cytokeratin 18 antibody (a marker for epithelial cells), quantitated by flow cytometry and only cultures that were >90% cytokeratin positive were used for experiments.

The surface expression of TRAIL receptors was measured by flow cytometry, using goat polyclonal antibodies against the extracellular domain of the respective receptors.

Immunohistochemistry was performed on paraffin-embedded sections from normal thyroid glands and Hashimoto's thyroiditis to investigate the *in vivo* expression of TRAIL and its receptors. Immunocytological staining was also done on cultured thyroid cells to detect DR5 and DcR1 expression and was analyzed by light and confocal microscopy.

The proteasome activity in the lysate of cultured thyroid cells was determined by fluorogenic peptide substrate assay and was standardized to the hydrolysis of Suc-LLVY-AMC by 5 μg recombinant proteasome as a positive control (100%).

Induction of EAT: Eight-week-old female CBA/J mice, a strain susceptible to EAT, were s.c. challenged with 100 μg of porcine Tg emulsified in complete Freund's adjuvant. Two weeks later the mice were boosted with the same dose of pTg in incomplete Freund's adjuvant. One week later mice were i.p. injected with 5 μg mouse recombinant IFNγ, 0.5 μg mouse recombinant TNFα or the combination of them in PBS for 3 consecutive days.

Anti-Tg antibodies were assayed by ELISA.

Thyroid histopathology in EAT: The lymphocytic infiltration of thyroids was evaluated on H&E stained slides. The severity of thyroiditis was graded on a scale of 0–4. Apoptosis in thyroid sections was detected by TUNEL staining of fragmented DNA. The analysis of infiltrating cells was made by immunohistochemistry, using anti-CD45, anti-CD4 and anti-CD8 antibodies.

The function of the Fas pathway *in vivo* was analyzed in cytokine pretreated mice by injecting an agonist anti-Fas antibody directly into the thyroid. Eight hours after the procedure mice were sacrificed and thyroid tissues were harvested for analysis of apoptosis.

Engraftment of human thyroid tumors into SCID mice: Tumor tissue samples were obtained from ten patients with suspected thyroid malignancy during operation. One 8x4x3 mm sample from each thyroid tumor was cut into two pieces of identical size and transplanted into two C.B-17-SCID/SCID mice. After 16 weeks, the animals were sacrificed and organs of the animals were checked for the presence of local and distant metastases. The size and weight of each removed tumor implant were recorded.

Reconstitution of the human immune system: In the case of each tumor, one of the two mice has been injected intraperitoneally with $2x10^7$ peripheral blood mononuclear cells separated from the blood of the same tumor patient on day 1 postoperatively by Ficoll-Hypaque density gradient centrifugation.

Murine IgM and human IgG were measured by ELISA.

Histopathology in the SCID mouse model: The original human tumors (before transplantation), the explanted thyroid tissues and the organs of mice were investigated using histology and, in the cases of DTC immunohistochemistry. Monoclonal antibodies to human markers included anti-CD3, anti-CD8, anti-CD20, anti-CD45RO, anti-CD68, anti-HLA-DR and anti-thyroglobulin.

Computer software and statistical analysis: Flow cytometry data were analyzed by WinMDI 2.8. Densitometry of autoradiograms was done using Quantity One. Statistical analysis was performed using χ^2-test, Student's t-test and Wilcoxon matched pairs test using Stat View software.

RESULTS

1. Normal human thyroid cells *in vitro* were resistant to TRAIL but the combination of IL-1β and TNFα induced susceptibility to TRAIL-mediated apoptosis, with lower but significant levels of cell death detectable in IL-1β pretreated cultures. No cell death was observed with TNFα pretreatment. The additional pretreatment of these cultures with IFNγ inhibited cell death in both circumstances. TRAIL-mediated cell death occurred after three days of cytokine pretreatment with maximum detectable death after four days pretreatment. Near maximum cell death was demonstrated within 4h after TRAIL addition and TRAIL was effective even at 50 ng/ml concentration. Soluble DR5 blocked the cell death while soluble TNF-R1 does not, verifying that the primary death signal was provided by TRAIL.

2. The analysis of the expression of TRAIL receptors in lysates of cultured thyroid cells indicated that DR5 is regulated by cytokines at posttranscriptional level. The total protein and cell surface expression of DR5 was increased by IL-1β and TNFα/IL-1β pretreatment and correlated with the susceptibility to TRAIL. DcR1 mRNA and cell surface expression was suppressed by TNFα/IL-1β, in agreement with its potential role as an inhibitor of TRAIL-mediated apoptosis. Changes in the mRNA and protein levels of DR4 and DcR2 did not show any relationship with TRAIL susceptibility. Exposure of cytokine pretreated TECs to an agonist anti-DR5 antibody revealed that the apoptotic signal was transmitted by DR5.

3. TRAIL and all TRAIL receptors were expressed in thyroid cells *in vivo* in normal thyroid tissue and in samples from HT. TRAIL expression was markedly increased in thyrocytes from HT while was not detected in infiltrating lymphocytes. However, a significant portion of the infiltrating lymphocytic cells in HT expressed DR5 and DcR1.

4. The experimentally induced autoimmune thyroiditis in mice is self-limited and is not accompanied by the disruption of follicular structure and hypothyroidism, so it is not identical with human Hashimoto's thyroiditis. In order to improve this model, proinflammatory cytokines were used in addition to immunization with porcine thyroglobulin. The addition of IFNγ/TNFα significantly sustained the lymphocytic infiltration in the thyroid of pTg immunized mice and resulted in the destruction of follicular architecture, without altering the humoral immune response. An increase in thyroid cell apoptosis was also detected. The potential

involvement of Fas-mediated apoptosis in this process was investigated by direct injection of agonist anti-Fas antibody into the thyroid of mice. The number of apoptotic thyroid epithelial cells was markedly increased in mice pretreated with IFNγ/TNFα compared with mice treated with Fas agonist alone.

5. Primary thyroid cells from well-defined goiter nodules were resistant to TRAIL and Fas-mediated apoptosis.

6. In contrast to normal cells, the majority of goiter-derived cells remained resistant to TRAIL and/or agonist anti-Fas antibody after cytokine pretreatment. The sensitivity to TRAIL-induced cell death inversely correlated with the goiter size.

7. The resistance of goiter cells to TRAIL was not regulated on the level of death and decoy receptors and was not explained by changes in the concentration of known intracellular inhibitors of apoptosis.

8. Normal thyroid cells were characterized by low proteasome activity. Lysates from goiter cell cultures resistant to both death ligands demonstrated proteasome activity that was significantly increased over normal thyroid cells and was similar to the proteasome activity observed in papillary cancers. In contrast, death ligand sensitive goiters and goiters resistant to only one of the two death ligands showed low proteasome activity, similar to normal cells. Proteasome inhibitors could sensitize resistant goiter cells to TRAIL-mediated apoptosis.

9. The hu-PBL-SCID mouse model was established to study human thyroid tumors *in vivo.* It was found that papillary, follicular, anaplastic and medullary cancers could successfully be grown in SCID mice. Growth rate, as expected, was more rapid in undifferentiated cancers. The model seemed to be appropriate to investigate the biological characteristics of human thyroid cancers. However, the reconstitution of the human immune system was not complete in mice received human peripheral blood mononuclear cells. At the same time tumor infiltrating lymphocytes were able to unintentionally reconstitute a certain level of human immune system.

CONCLUSIONS AND NEW FINDINGS

1. TRAIL-mediated apoptosis of thyroid epithelial cells is regulated by inflammatory cytokines. IL-1β alone and in combination with TNFα induces sensitivity to TRAIL, while the further addition of IFNγ makes these cells resistant to apoptosis induced by TRAIL. This is the first reported demonstration of IL-1β induction of TRAIL sensitivity and inhibition of TRAIL sensitivity by IFNγ.

2. This pattern of TRAIL sensitivity correlates with levels of cell surface expression of TRAIL receptors. IL-1β/TNFα enhancement of DR5 surface expression provides a mechanism for TRAIL sensitivity. IFNγ counteracts this activity by suppressing DR5 surface expression, thus preventing apoptotic signal transduction. It was documented by the use of DR5 specific agonist antibody that the death signal is mediated through DR5. These data are the first to demonstrate the induction of cell surface DR5 by IL-1β alone or in synergy with TNFα. This is also the initial demonstration of regulation of DcR1 cell surface expression levels by any treatment of any cell type especially primary cells.

3. We also show for the first time the *in vivo* expression of TRAIL and its receptors in the thyroid gland in both normal follicles and follicles undergoing autoimmune destruction. The presence of all these proteins in normal thyroids supports that the TRAIL pathway is tightly regulated and is normally inhibited. The regulation of the TRAIL apoptosis signaling pathway by the inflammatory cytokines TNFα, IL-1β and IFNγ, which are known to be present in inflamed thyroids, suggests a role for these proteins in autoimmune thyroiditis.

4. It was presented for the first time that the combination of IFNγ and TNFα prolonged the lymphocytic infiltration and induced follicular disruption in murine EAT, possibly through the Fas pathway. This may help to explain the differences between EAT and HT, because the thyroid in patients with HT has a chronic inflammatory environment enriched in Th1 cytokines such as IFNγ and TNFα. These data suggest that other disorders with Th1 immune response may result in cellular apoptosis through the Fas-FasL pathway due to the cytokine environment. Genetic differences in the cytokine regulation of this pathway

involving apoptosis regulatory proteins may predispose individuals to autoimmune disorders.

5. It is an original observation, that goiter-derived thyroid cells are resistant to TRAIL and/or Fas-mediated apoptosis *in vitro* and cannot be sensitized by inflammatory cytokines. This represents a new aspect of aberrant growth regulation in goiter nodules. The inverse correlation of goiter size with the sensitivity to TRAIL-mediated apoptosis suggests a relationship to goiter pathogenesis.

6. The increased proteasome activity in a subset of resistant goiters and the ability of proteasome inhibitors to sensitize resistant goiter cells to TRAIL suggests that the proteasome is an important regulator of apoptosis in goiter nodules. This is the first study of proteasome activity in normal and goiter-derived thyroid cells and the first demonstration of a positive correlation between the proteasome activity and resistance to death receptor-mediated apoptosis.

7. In order to examine anti-tumor immunity and the regulation of apoptosis in differentiated thyroid tumors in forthcoming *in vivo* studies, the hu-PBL-SCID mouse model was established. This model is adequate to investigate the biological characteristics of thyroid cancers but warrants improvement to analyze the interaction between the immune system and tumor cells.

FUTURE PERSPECTIVES

Clarifying the regulation of apoptosis signaling provides an opportunity to influence disease progression in autoimmune thyroiditis. The unique and exclusive combinations of cytokines sensitizing to TRAIL- and Fas-mediated apoptosis suggest that these complimentary activities have an important function and the cytokine milieu in the thyroid microenvironment can determine the activity of apoptosis pathways. The aberrant regulation of apoptosis in goiter nodules reveals a new aspect of goiter pathogenesis and warrants further studies. The high proteasome activity in resistant goiters and papillary cancers suggests a benefit from treatment with proteasome inhibitors (which are currently in clinical trials) in these disorders. The successful transplantation of human thyroid tumors into SCID mice allows us to test the *in vivo* sensitivity of primary tumors to TRAIL which is a promising anti-cancer agent, before clinical trials.

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