Chapter 16
History of Histamine-Releasing Factor (HRF)/ Translationally Controlled Tumor Protein (TCTP) Including a Potential Therapeutic Target in Asthma and Allergy

Susan M. MacDonald

Abstract Histamine-releasing factor (HRF) also known as translationally controlled tumor protein (TCTP) is a highly conserved, ubiquitous protein that has both intracellular and extracellular functions. Here we will highlight the subcloning of the molecule, its clinical implications, as well as an inducible-transgenic mouse. Particular attention will be paid to its extracellular functioning and its potential role as a therapeutic target in asthma and allergy. The cells and the cytokines that are produced when stimulated or primed by HRF/TCTP will be detailed as well as the downstream signaling pathway that HRF/TCTP elicits. While it was originally thought that HRF/TCTP interacted with IgE, the finding that cells not binding IgE also respond to HRF/TCTP called this interaction into question. HRF/TCTP or at least its mouse counterpart appears to interact with some, but not all IgE and IgG molecules. HRF/TCTP has been shown to activate multiple human cells including basophils, eosinophils, T cells, and B cells. Since many of the cells that are activated by HRF/TCTP participate in the allergic response, the extracellular functions of HRF/TCTP could exacerbate the allergic, inflammatory cascade. Particularly exciting is that small molecule agonists of the phosphatase SHIP-1 have been shown to modulate the P13 kinase/AKT pathway and may control inflammatory disorders. This review discusses this possibility in light of HRF/TCTP.

S.M. MacDonald, M.D. (✉)
The Johns Hopkins Asthma and Allergy Center, 5501 Hopkins Bayview Circle, Room 3B.69, Baltimore, MD 21224, USA

Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA
e-mail: smacdona@jhmi.edu

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16.1 Introduction/Cloning

Histamine-releasing factor (HRF) was originally classified as a tumor protein (translationally controlled tumor protein, TCTP) in both mouse acidic tumor and mouse erythroleukemia. Brawerman's group in the 1980s named the protein, but its function remained a mystery (Yenofsky et al. 1983; Chitpatima et al. 1988). We identified a histamine-releasing activity that was found in late-phase fluids from nasal lavages, bronchoalveolar lavage fluids (BAL), and skin blister fluids that directly induced histamine release from basophils isolated from a subpopulation of allergic donors (HRF-responders [HRF/TCTP-R]) (MacDonald et al. 1987b). By definition, donors with basophils who did not directly respond to HRF/TCTP were termed HRF-non-responders (HRF/TCTP-NR).

Although an IgE-dependent HRF can be detected in nasal lavages (MacDonald et al. 1987b), PBMC culture supernatants (Sampson et al. 1989), and fluids from human late-phase reactions (LPR) (MacDonald et al. 1987b), we used supernatants from overnight cultures of U937 cells, a human macrophage cell line (Sundstrom and Nilsson 1976), for the isolation and sequencing of the HRF. Fifty liters of these supernatants were concentrated, and the proteins were contained therein purified by Sephadex G75 gel filtration, MONO Q anion exchange, and repetitive Superdex chromatography. The basophil-releasing activity was concentrated, subjected to SDS-polyacrylamide gel electrophoresis (PAGE), blotted onto a polyvinylidene difluoride membrane, and stained with Coomassie blue, revealing four major protein bands (at 60 kDa and 29 kDa and a doublet at 23 kDa). The NH2-terminal sequences of each of these four bands were determined by protein sequencing. The 18 NH2-terminal amino acids of one of the 23-kDa components after a GenBank search revealed 94% homology to p2l, a predicted 21-kDa murine peptide whose complementary DNA (cDNA) was isolated from mouse tumor cells (MacDonald et al. 1987b), as well as identity to p23, the human homolog, described by Bohm et al. (1989). Both were cloned on the basis of their abundant expression in tumor cells, and no function has been ascribed to either molecule. Because there is a stop codon upstream from the initial methionine, it appears that p2l and p23 are not posttranslationally processed at their NH2-termini. p21 cDNA was subcloned into the pGEX-2T plasmid (Smith and Johnson 1988), expressed as a fusion protein with glutathione-S-transferase (GST), purified, and isolated from GST by cleavage with thrombin. Due to the homology between p2l and p23, the same synthetic primers, based on the mouse p2l sequence, were used to isolate the human p23 cDNA from the U937 cell line. This protein also was expressed in *Escherichia coli* as a GST fusion protein and was subsequently cleaved from GST with thrombin.

After purification and cloning, HRF was found to be identical to TCTP, which is also known as p23 (MacDonald et al. 1995). Our recombinant molecule was found to have the same properties and ability to induce histamine release from selected donors as did the originally described HRF/TCTP derived from nasal secretions. The protein is ubiquitously expressed as an intracellular protein, and homologs of HRF/TCTP are found in parasites including *Plasmodium falciparum*, *Wuchereria*

bancrofti, *Brugia malayi*, and *Schistosoma Mansoni*. All of these parasites possess mast cell/basophil histamine-releasing activity (MacDonald et al. 2001; Gnanasekar et al. 2002; Rao et al. 2002). Our group, as well as another group, has identified the interaction between HRF and elongation factor-1δ, also known as eukaryotic elongation factor 1B-β (Langdon et al. 2004; Cans et al. 2003). Thus, HRF/TCTP may have an intracellular role in interfering with the elongation step of protein synthesis.

16.2 Clinical Relevance of HRF/TCTP

The history of HRFs dates back to 1979 when Thueson et al. (1979) first described a histamine-releasing activity produced by cultured peripheral blood mononuclear cells that had been stimulated with mitogens or antigens. This HRF was further characterized and found to be very heterogeneous, containing molecular weight species ranging from 15,000 to 50,000 kDa. A number of other groups confirmed this finding (reviewed in MacDonald et al. 1987b). HRFs are produced in vitro by a variety of cell types such as T and B lymphocytes, mononuclear cells, alveolar macrophages, platelets, vascular endothelial cells, and various cell lines, including the U937 monocyte/macrophage-like cell line and RPMI 8866 B-cell line. Not only is HRF found in vitro, but it is also found in vivo.

HRF/TCTP's link to human asthmatic, allergic disease has been well accepted. It has been found in human respiratory secretions (BAL) and skin blister fluids (MacDonald et al. 1987b). Since not all donors' basophils release histamine when exposed to HRF/TCTP, we undertook a study to define the responding population. Sixty-four ragweed allergic patients with a history of seasonal rhinitis and one or more positive skin tests were compared to 17 nonatopic controls who were skin test negative. Sensitivity to HRF/TCTP was restricted to a subpopulation of atopic individuals (MacDonald et al. 1987a). In a separate study of 55 ragweed allergic patients, there was a significant correlation between the intensity of symptoms in the late-phase reaction and basophil histamine release to HRF/TCTP (MacDonald 1993). In studies from another group, peripheral blood mononuclear cells from patients with asthma spontaneously produced HRF/TCTP (Alam et al. 1984; Alam and Rozniecki 1985). That production of HRF/TCTP not only correlated with bronchial hyperreactivity but the bronchial sensitivity to methacholine of the patient correlated with the magnitude of HRF/TCTP production (Alam et al. 1987). Sampson et al. have shown that the production of HRF/TCTP also is associated with clinical status of food allergy and atopic dermatitis (Sampson et al. 1989). Using blood from food allergic children with atopic dermatitis, they found that their basophils have a high spontaneous release of histamine and their cultured mononuclear cells spontaneously produce HRF/TCTP. When these children were placed on an avoidance diet, they improved clinically, their basophils no longer spontaneously secreted histamine, and their mononuclear cells no longer spontaneously produced HRF/TCTP. Two groups have reported the effects of immunotherapy on HRF/TCTP production. One group showed a striking correlation between the production of

HRF/TCTP by mononuclear cells and the change in bronchial sensitivity to histamine (PC20) after 2 years of immunotherapy (Kuna et al. 1989). Brunet et al. showed immunotherapy in allergic rhinitis patients without asthma improved symptoms and also avoided the seasonal increase of spontaneous and antigen-driven HRF/TCTP production from peripheral blood mononuclear cells (Brunet et al. 1992). Moreover, we have measured HRF in human BAL fluids of allergics following antigen challenge. While HRF/TCTP increases over baseline after antigen challenge, it is not significant with the number of patients ($n = 8$) we have investigated (unpublished observations).

With the availability of recombinant material, we examined the lymphocytes of allergic and nonallergic patients for the generation of HRF/TCTP mRNA and protein. Twelve patients (four HRF/TCTP-R, four HRF/TCTP-NR, and four nonallergic) were recruited. Blood was drawn for serum IgE measurements and for basophil histamine release in response to recombinant HRF/TCTP and anti-IgE. In addition, peripheral blood mononuclear cells were cultured for HRF/TCTP production and processed for mRNA extraction and subsequent reversed transcribed polymerase chain reaction for HRF/TCTP mRNA. The geometric mean serum IgE levels were 356 ng ml^{-1} in the HRF/TCTP-R group versus 52 μg ml^{-1} and 4.2 μg ml^{-1} in the HRF/TCTP-NR and nonallergic subjects, respectively. Histamine release in response to the recombinant HRF/TCTP paralleled that of our native HRF/TCTP preparation in that only the four HRF/TCTP-R patients released histamine to this stimulus. The quantity of mRNA for HRF/TCTP, when compared to that for beta-actin, the housekeeping gene, did not appear different among the groups. The bioactivity of the recombinant HRF/TCTP on lactic acid-treated cells passively sensitized with an IgE containing serum from a HRF/TCTP-R, however, was greater in the allergic, HRF/TCTP-R patients than in the nonallergic subjects (MacDonald 1996; Langdon et al. 1995). Thus, it appears that all individuals make mRNA for HRF/TCTP, but atopic subjects more effectively translate it to protein. In an abstract, the serum from some patients with atopic dermatitis, but not normals, demonstrated increased levels of HRF/TCTP-reactive IgE levels (Ando et al. 2012). These atopic dermatitis patients' sera could cause cytokine secretion from human mast cells (Ando et al. 2012).

16.3 HRF/TCTP Extracellular Functions

Secreted by an ER/Golgi-independent route, HRF/TCTP has no leader sequence, as documented by Amzallag et al. (2004). They discerned that secreted HRF/TCTP comes from an existing intracellular pool and co-distributes with TSAP6, a member of a family that is involved in vesicular trafficking and secretory processes (Amzallag et al. 2004; Moldes et al. 2001; Korkmaz et al. 2002). Our focus has been on the extracellular functions of HRF/TCTP. HRF was initially described as a complete secretagogue for histamine and IL-4 secretion from basophils of allergic donors (Schroeder et al. 1996). These donors were thought to have a certain type of

IgE that interacted with HRF/TCTP to induce secretion (MacDonald et al. 1995). However, it was subsequently demonstrated that HRF/TCTP primed all basophils for histamine release, IL-4 and IL-13 secretion regardless of the type of IgE (Schroeder et al. 1997). Additional studies demonstrated that HRF/TCTP did not appear to interact with IgE. Namely, pharmacologic agents that altered HRF/TCTP-induced histamine release, i.e., rottlerin, did not affect anti-IgE-induced histamine release (Bheekha-Escura et al. 1999). Rat basophilic leukemia cells transfected with the α, β, and γ chains of the human IgE receptor, FcεRI, did not release histamine to HRF/TCTP despite sensitization with IgE molecules from an HRF/TCTP-R donor (Wantke et al. 1999). HRF/TCTP was shown to stimulate eosinophils to produce IL-8 and induce an intracellular calcium response (Bheekha-Escura et al. 2000). This was also observed in the eosinophil cell line, AML-3D10, which does not express the α chain of the FcεR1 on the surface of the cell (Bheekha-Escura et al. 2000). Very recently, HRF/TCTP was found to have an inflammatory role in mouse models of asthma and allergy, whereby HRF/TCTP was found to exist as a dimer, bound to a subset of IgE and IgG antibodies by interacting by its N-terminus and some internal regions with the Fab region of immunoglobulins (Kashiwakura et al. 2012). These interactions were described with mouse HRF/TCTP and interacted on mouse mast cells.

At the level of gene transcription, HRF/TCTP has been shown to inhibit cytokine production from stimulated primary T cells and the Jurkat T-cell line (Vonakis et al. 2003). Thus, HRF/TCTP, in addition to functioning as a histamine-releasing factor, can modulate secretion of cytokines from human basophils, eosinophils, and T cells. It has also been identified as a B-cell growth factor by Kang et al. They demonstrated that HRF/TCTP bound to B cells and induced cytokine production (Kang et al. 2001). More recently, HRF/TCTP was shown to stimulate bronchial epithelial cells to produce IL-8 and GM-CSF (Yoneda et al. 2004). These effects of HRF/TCTP on different cell types are depicted in Fig. 16.1.

16.4 Other Functions of HRF/TCTP (Mainly Intracellular)

While this review focuses mainly on the extracellular functions of HRF/TCTP, it is important to discuss some of its broad spectrums of intracellular functions. HRF/TCTP is both transcriptionally and posttranscriptionally regulated by calcium (Xu et al. 1999). It is also a tubulin-binding protein and has been shown to transiently associate with microtubules during the cell cycle (Gachet et al. 1999). Also the vitamin D receptor, the NF-κβ regulatory subunit, Iκκγ(NEMO), the myeloid cell leukemia protein 1 (MCL1), and Bcl-XL have been demonstrated to interact with HRF/TCTP (Rid et al. 2010; Fenner et al. 2010; Zhang et al. 2002; Yang et al. 2005). High levels of HRF/TCTP have been associated with various cancers, such as prostate, breast, and colon cancer (Arcuri et al. 2004; Vercoutter-Edouart et al. 2001; Chung et al. 2000). Furthermore, the gene for HRF/TCTP was downregulated in tumor reversion, and more specifically, the level was significantly

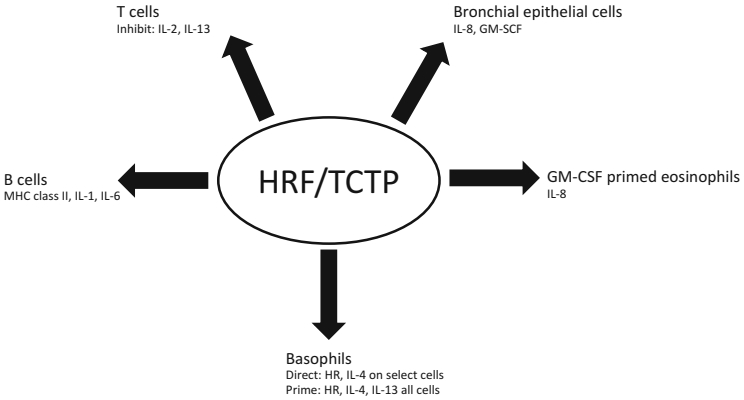

Fig. 16.1 Effects of HRF/TCTP on various cell types. HRF/TCTP either directly activates (direct) basophils producing HR and IL-4 on certain cells or primes (prime) anti-IgE-induced HR and IL-4 and IL-3. HRF/TCTP induces IL-8 from GM-CSF-primed eosinophils. Similarly, it produces IL-8 and GM-CSF from bronchial epithelial cells and MHC class II, IL-1, and IL-6 from B cells. Contrary to the enhanced interleukin production, HRF/TCTP inhibits IL-2 and IL-12 from T cells. Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; HR, histamine release; HRF/TCTP, histamine-releasing factor/translationally controlled tumor protein; IL, interleukin; MHC, major histocompatibility complex

reduced in a lung cancer cell line, A549, and revertant cells (Tuynder et al. 2004). This was eloquently described by Telerman and Amson in a publication entitled *The Molecular Programmme of Tumors Reversion: The Steps Beyond Malignant Tumor Formation* (Telerman and Amson 2009). The role of HRF/TCTP in tumor development may be associated with its antiapoptotic activity (Yang et al. 2005; Li et al. 2001). This is further supported by reports of HRF/TCTP antagonizing Bax function and controlling the stability of the tumor suppressor p53 (Susini et al. 2008; Rho et al. 2011). In a very recent publication, HRF/TCTP promoted p53 degradation, and p53 directly repressed HRF/TCTP transcription (Amson et al. 2011). With this report of a previously unrecognized regulatory circuit, HRF/TCTP may be extremely relevant in cancer (Amson et al. 2011). As previously mentioned, our lab and others have shown involvement of HRF/TCTP in the elongation step of protein synthesis (Langdon et al. 2004; Cans et al. 2003). Thus HRF/TCTP's intracellular functions are wide ranging. The extracellular functions, however, seem to focus on inflammation.

16.5 An Inducible HRF/TCTP Transgenic Mouse

Although HRF/TCTP has been extensively investigated for many years, most studies have been carried out in cultured cells and pathologic samples. Until recently, there has been no established animal model available to explore the

function of HRF/TCTP. Several groups generated HRF/TCTP knockout mice by targeted gene disruption, but these HRF/TCTP knockout mice were embryonically lethal (Susini et al. 2008; Chen et al. 2007). There has been a TCTP mouse generated by Telerman and colleagues (Thébault et al. 2016). Since HRF/TCTP is ubiquitous and highly conserved, our approach was to create an inducible HRF/TCTP mouse model using the tet-on system. We wanted to target HRF/TCTP to the lungs, so we used the CC10 promoter that is expressed in Clara cells of the lung epithelium to generate a transgenic TRE-HRF-EGFP mouse. The HRF transgenic plasmid was generated by the combination of three main components (Fig. 16.2). The first component is the pTRE-tight vector (provided by Dr. Zhu in our division), which contains a modified TRE (tetracycline response element) controlling the inducible expression of the gene of interest. The second component is human HRF cDNA, which was cloned from U937 cells by RT-PCR and further confirmed by sequencing. The third component is the pIRES2-EGFP vector, provided by Dr. Vonakis in our division. The IRES2 (internal ribosome entry site) allows the EGFP (enhanced green fluorescent protein) gene to be expressed individually as a reporter protein along with HRF in order to facilitate the recognition of expression of transgenic human HRF. Thus, the transgenic TRE-HRF-EGFP construct will express HRF and EGFP individually under the regulation of tetracycline or doxycycline. Using this model, we saw an enhanced asthmatic, allergic phenotype after OVA challenge (Yueh-Chiao et al. 2010). This enhancement is in the C57BL/6 mouse, not the traditional "allergic" BALB/c mouse. The development of an inducible-transgenic HRF/TCTP animal model will yield insights into its underlying pathophysiologic characteristics and provide a tool to define the mechanism of this enhanced or primed phenotype.

The mechanism of HRF/TCTP's enhanced response yielding increases in IL-4, IgE, and eosinophils after OVA challenge in our transgenic model is currently unknown. All of these events that could be attributed to the action of HRF/TCTP on the basophil is plausible considering the data on HRF/TCTP and the human basophil. We have shown that HRF/TCTP activates human basophils to produce IL-4 (Schroeder et al. 1996). It is well accepted that IL-4 is important for B-cell class switching and production of IgE. Furthermore, human basophils possess the β-1 integrin that is important for firm adhesion. The ligand for β-1 integrin, vascular cell adhesion molecule (VCAM-1) is upregulated by IL-4 and is important for the transendothelial migration of eosinophils and Th2 cells (Schroeder 2009). Therefore, the production of IL-4 by basophils could explain the enhanced asthmatic, allergic phenotype we see after overproduction of HRF/TCTP in our OVA-challenged model. This, of course, assumes that the mouse basophil acts in a similar manner as its human counterpart. While the existence of the mouse basophil dates back over two decades, this cell has reemerged in the last several years as an important initiator in mouse Th2 inflammation (Seder et al. 1991; Min and Paul 2008; Obata et al. 2007).

The multifactorial disease that is asthma makes it highly unlikely that one single cell such as the mouse basophil is solely responsible for HRF/TCTP's effect on asthmatic lung disease. Furthermore, one must consider eosinophils and T cells. It is well known that the activation, recruitment, and proliferation of the T cell are

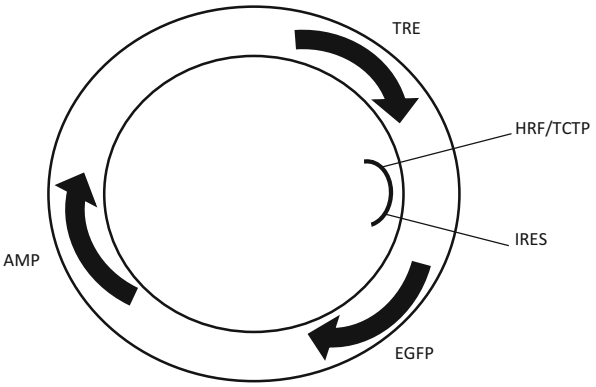

Fig. 16.2 Schema of the transgenic TRE-HRF/TCTP- EGFP plasmid construction. The human HRF gene was inserted between the TRE and IRES-EGFP elements. Abbreviations: TRE, tetracycline response element; HRF/TCTP, histamine-releasing factor; IRES, internal ribosome entry site; EGFP, enhanced green fluorescent protein

associated with asthmatic lung disease (Jacobsen et al. 2008). Given the HRF/TCTP's activation of eosinophils and the increased eosinophils we find in the BAL fluid of the OVA-challenged transgenic mouse, it is logical to examine the role of this cell in the mechanisms of action of HRF/TCTP in vivo (Bheekha-Escura et al. 2000; Yueh-Chiao et al. 2010). In the congenitally eosinophil-deficient PHIL mouse, there is a diminution of Th2 responses (Lee et al. 2004). Furthermore, eosinophils also secrete IL-4 and act as antigen-presenting cells yielding T-cell activation after allergen provocation in the lung (Obata et al. 2007; Mayr et al. 2002). Therefore, HRF/TCTP may exert additional enhancing effects through the eosinophil. Since an antibody to IL-5 has been shown to suppress eosinophil recruitment following OVA challenge in WT and FcεRα-/- mice (Mayr et al. 2002), giving anti-IL-5 to our OVA-challenged HRF/TCTP mice could help determine HRF/TCTP's mechanism upon eosinophil recruitment. Alternately, cross-breeding our HRF/TCTP transgenic mice with the eosinophil knockout PHIL mouse could ablate all HRF/TCTP-induced enhancing effects or just affect eosinophils. Future possibilities are many using this model as a tool.

16.6 The Importance of Ship-1 on HRF/TCTP Signaling

That human basophils are cells capable of being "primed" or having an enhanced functional response has long been appreciated. Some of the molecules that are known to prime human basophils include IL-3, NGF, HRF/TCTP, and the nonphysiologic stimulus D_2O (Schroeder 2009; MacDonald et al. 1989, 1991). In general, these substances show a greater *releasability* (as evidenced by histamine or IL-4 secretion) when stimulating basophils from allergic or allergic/asthmatic subjects. They do not generally activate basophils from normal subjects. The exception to this is the HRF/TCTP-R basophils. Basophils from these subjects are directly activated by D_2O, IL-3, and HRF/TCTP (MacDonald et al. 1989, 1991).

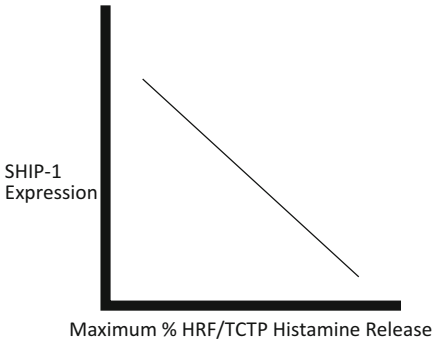

Fig. 16.3 Negative correlation between SHIP-1 protein expression and histamine release to HRF/TCTP. Histamine release was performed on the same day as SHIP-1 measurements

The molecular basis for the *releasability* of the HRF/TCTP's basophils remained elusive until relatively recently. It has become accepted that the term *releasability* (i.e., control of release of mediators from basophils in response to different stimuli) involves several biochemical events in addition to the surface density of IgE molecules. There have been reports of certain signaling molecule deficiencies in nonreleasing basophils (Kepley et al. 1999; Lavens-Phillips and MacGlashan 2000). While these deficiencies are documented, there is little variation of SHIP-1 in the general population (Vilariño et al. 2005). To date, we are the first group to show the negative association of the phosphatase, SHIP-1, with histamine release to HRF/TCTP in *hyperreleasing* basophils (Vonakis et al. 2001). See Fig. 16.3. Variation of SHIP-1 levels is also documented in a subset of patients with chronic idiopathic urticaria, where levels of SHIP-1 are increased and anti-IgE-induced histamine release is reduced (Vonakis et al. 2007). Thus, SHIP-1 levels appear to be altered in some human disease states.

A clue to the underlying mechanisms of increased *releasability* of basophils was demonstrated in a mouse knockout of SHIP-1 (Krystal 2000; Huber et al. 1998). Mast cells grown from the bone marrow (BMMC) of SHIP-1 knockout mice showed decreased hydrolysis of phosphatidylinositol (PI)-3,4,5,P_3 (PIP_3) (Huber et al. 1998). SHIP-1 participates in the pathway in which the lipid phosphatidylinositol 4,5 bisphosphate (PI4,5,P_2) is phosphorylated by PI3 kinase to produce PIP_3 which can be acted upon to produce PI(3,4)bisphosphate (PI3,4P_2) (Rohrschneider et al. 2000; Scharenberg and Kinet 1998). We have demonstrated that the compound, LY294002, an inhibitor of PI3 kinase, inhibits histamine release induced by HRF/TCTP in basophils from HRF/TCTP-R donors (Vonakis et al. 2001). The activity of PI3 kinase is central to many basophil functions, and SHIP-1 acts to oppose the function of PI3 kinase by removing the 5′phosphatase from PIP_3, making SHIP-1 an important regulator of these reactions. Mouse SHIP-1 knockout mast cells had an excess of PIP_3 that resulted in a sustained calcium signal that was critical for degranulation (Lioubin et al. 1996). Furthermore, SHIP is a suppressor of IgE plus antigen-induced degranulation of not only bone marrow-derived mast cells but also negatively regulates IgE plus antigen-induced degranulation of connective tissue and mucosal mast cells by repressing the P13 kinase pathway (Ruschmann

et al. 2012). Additionally, PIP_3 recruits the serine tyrosine kinase, Akt, to the plasma membrane (Brauweiler et al. 2000), which is present in human basophils and transiently phosphorylated after anti-IgE stimulation (Miura et al. 2001). Akt is phosphorylated by HRF/TCTP in HRF/TCTP-R donors but not in HRF/TCTP-NR donors (Vonakis et al. 2008). Furthermore, we see prolonged Akt phosphorylation kinetics in HRF/TCTP-R (Vonakis et al. 2008), which is supportive of the involvement of this pathway in HRF-induced activation. Data from the SHIP-1 knockout mice and our own published data suggest that SHIP-1 may play a "gatekeeper role" in mouse and human basophils and mast cells. One would expect SHIP-1 to limit effector cell responsiveness in normal individuals, while a SHIP-1 deficiency would predispose an individual to excess inflammatory-mediator production and, hence, a *hyperreleasable* phenotype.

In order to address this more directly, we altered SHIP-1 levels in human basophils. These studies have been limited by the fact that the basophil is an end-stage nondividing cell and extremely difficult to transfect or transduce. Many attempts have been tried to transfect primary human basophils. These include lipid-based reagents, lentivirus, and nucleofection. Most failed either due to toxicity or very low transfection efficiency. Only nucleofection (Amaxa) gave a limited transfection efficiency that was useful only for single-cell analysis (Vilarino and MacGlashan 2005). There is one report that a TAT-fusion protein was used in transfecting human basophils (Didichenko et al. 2008). We set out to determine a more efficient method of altering signal transduction pathways in human basophils. To that end, we established a model of culturing human peripheral blood-derived basophils from CD34+ cells that have the morphologic and functional characteristics of human basophils (Langdon et al. 2008). We utilized this model to alter SHIP-1 levels using siRNA technology and demonstrated a decrease in SHIP-1 levels that was associated with an increase in histamine release to HRF/TCTP. Using CD34+ peripheral-derived basophils, it is possible to perform a more direct test of the hypothesis that SHIP-1 has a role in modulating basophil responsiveness, both to HRF/TCTP and IgE-mediated stimulation.

16.7 Additional Intracellular Signaling by HRF/TCTP

Another possible mechanism of action for HRF/TCTP may be IgE-dependent enhancement. Originally, HRF/TCTP was called the IgE-dependent HRF (MacDonald et al. 1995). This designation resulted from the fact that HRF seemed to act as a secretagogue for human basophils from a subpopulation of allergic donors. Moreover, passive sensitization of serum containing IgE from these responding donors rendered nonresponsive donors' basophils responsive to HRF/TCTP (MacDonald et al. 1995). HRF/TCTP was then shown to activate other cells that do not possess the high-affinity IgE receptor, FcεR1 (Bheekha-Escura et al. 2000; Vonakis et al. 2003). We have demonstrated that HRF/TCTP has signal transduction events that are similar, but not identical, to signaling through FcεR1 (Vonakis et al. 2008). With the availability of

both the FcεR1α knockout mouse and the IgE knockout mouse (Dombrowicz et al. 1993; Oettgen et al. 1994), the question of whether HRF/TCTP is dependent on IgE can be definitively addressed. As mentioned, a manuscript has very recently been published that demonstrates mouse HRF/TCTP does bind to certain IgE and IgG molecules (Kashiwakura et al. 2012).

In order to address the molecular mechanisms of HRF/TCTP-induced secretion, we designed experiments to elucidate specific actions of HRF/TCTP on human basophils and to characterize the nature of intracellular signaling that follows stimulation with HRF/TCTP. Given the similarities in secretion kinetics following IgE-mediated stimulation, we hypothesized there would be some signaling characteristics similar to those previously found for IgE-mediated release. However, due to the differential sensitivity to treatment with rottlerin between HRF/TCTP and anti-IgE (Wantke et al. 1999), we also expected differences in signaling. We used human basophils from two donor populations, HRF/TCTP-R and HRF/TCTP-NR. Consistent with the ability of HRF/TCTP to either induce secretion directly from HRF/TCTP-R basophils or prime HRF/TCTP-NR basophils, we have shown binding of HRF/TCTP by flow cytometry to both donor populations (Vonakis et al. 2008). We demonstrated that HRF/TCTP induced activation of intracellular signal transduction events in basophils only from those donors who directly release histamine to HRF/TCTP, namely, HRF/TCTP-R. Specifically, we have been able to demonstrate increases in the arachidonic acid metabolite, LTC_4, from basophils of HRF/TCTP-R donors stimulated with anti-IgE. Additionally, we have demonstrated LTC_4 release from basophils stimulated with HRF/TCTP (Vonakis et al. 2008). One might predict that this might well be due to prolonged phosphorylation of MEK and ERK 1/2. Using human basophils isolated from leukopheresis packs, Miura et al. have demonstrated that the activation of ERK1/2 is linked to arachidonic acid metabolism but not to histamine or IL-4 release (Miura et al. 1999). Phosphorylation of ERK1/2 is transient, peaks at 5 min, and returns to baseline by 30 min. We have demonstrated that both MEK and ERK1/2 are phosphorylated by HRF/TCTP in basophils from HRF/TCTP-R donors but not from HRF/TCTP-NR donors (Vonakis et al. 2008). Thus, the characteristics of the signaling responses were very similar to those observed for stimulation with anti-IgE antibody or antigen with a couple of exceptions. Notably, there was no phosphorylation of FcεR1γ, and there was absolutely no phosphorylation of any downstream signal transduction molecules in the HRF/TCTP-NR basophils.

16.8 HRF/TCTP as a Therapeutic Target

Based on the above observations, we believe that HRF/TCTP may be an important element of the pathogenesis of asthmatic, allergic diseases. Since HRF/TCTP is present in late-phase reaction fluids in vivo, it may be contributing to mediator release that is found in the late response. Therefore, it is most reasonable to consider HRF as a therapeutic target. The most direct way to prove that HRF/TCTP is a

therapeutic target would be to block its binding to its receptor. However, despite numerous attempts by different laboratories, the HRF/TCTP receptor has remained elusive. An HRF/TCTP-blocking antibody would prove useful in this approach. Unfortunately, no specific antibody exists. A recent publication does demonstrate that the extracellular actions of HRF/TCTP can be explained, at least in part by specific binding sequences on mouse HRF (mHRF) to some IgE and IgG molecules (Kashiwakura et al. 2012). In two regions, the N-terminal 19-residue peptide and residues 107–135, the H3 region, were found to be important for this binding to immunoglobulins (Kashiwakura et al. 2012). These regions overlapped only in part with the antigen binding site. Furthermore, only certain, but not all, IgE and IgE molecules supported or bounded to HRF/TCTP (Kashiwakura et al. 2012). Nevertheless, this observation warrants further investigation.

Two additional observations remain. The possibility exists that this HRF/TCTP-immunoglobulin interaction could be explained by nonspecific ionic interactions or interactions of different parts of the immunoglobulins. Moreover, BAL and sera from naïve mice contain HRF/TCTP that does not normally yield inflammation (Kashiwakura et al. 2012). This suggests there might be a suppressive mechanism of inflammation induced by endogenous HRF/TCTP. Our own SHIP data with the inverse correlation of levels of SHIP-1 protein with histamine release to HRF/TCTP would support this (Vonakis et al. 2001).

Miu, Ong, and colleagues have discovered small molecule agonists of SHIP-1 that inhibit the P13K pathway (Ong et al. 2007). These are potent and specific activators of SHIP-1. Initial mouse model studies suggested that these agonists might be useful therapeutically. Our laboratory received such an agonist and was able to demonstrate that anti-IgE-induced basophil histamine release was inhibited while F-met-leu-phe-induced release was not (data not shown). In fact, it has recently been reported at the American Thoracic Society Meeting in May 2012 in San Francisco that the SHIP-1 agonist, AQX-1125, from Aquinox Pharmaceuticals was tested in a three-part phase I study that included a single ascending dose, a multiple ascending dose, and a food-effect study in healthy human volunteers (Tam et al. 2012). The drug was well tolerated and had a half-life that supported a once-daily oral administration.

"Aquinox Pharmaceuticals is a clinical-stage pharmaceutical company discovering and developing targeted therapeutics in disease areas of inflammation and immune oncology. Our primary focus is anti-inflammatory product candidates targeting SH2-containing inositol-5′-phosphate 1, or SHIP1, which is a key regulator of an important cellular signaling pathway in immune cells, known as the P13K pathway. Our lead product candidate, AQX-1125, is a small molecule activator of SHIP1 suitable for oral, once daily dosing. Having successfully completed multiple preclinical studies and seven clinical trials with AQX-1125, they are now advancing towards pivotal Phase 3 trials with AQX-1125, in our lead indication of bladder pain syndrome/interstitial cystitis (BPS/IC). Aquinox has a broad intellectual property portfolio and pipeline of preclinical drug candidates that activate SHIP1 (www.aqxpharma.com)." It should be noted that AQX-1125 was used to determine its ability to reduce symptoms exacerbations in COPD. The results of that trial called

FLAGSHIP was reported in July 2015 and demonstrated no difference between AQX-1125 and placebo, and therefore further development of AQX-1125 for COPD has been suspended and redirected their efforts into bladder pain syndrome/intestinal cystitis (BAS/IC) (www.aqxpharma.com).

"AQX-1125, Aquinox's lead drug candidate, is a small molecule activator of SHIP1, which is a regulating component of the P13K cellular signaling pathway. By increasing SHIP1 activity, AQX-1125 accelerates a natural mechanism that has evolved to maintain homeostasis of the immune system and reduce the immune cell activation and migration to sites of inflammation. AQX-1125 has demonstrated preliminary safety and favorable drug properties for once daily oral administration in multiple preclinical studies and seven completed clinical trials. Aquinox completed a successful Phase 2 clinical trial with AQX-1125 for the treatment of IC/BPS in the third quarter of 2016 (www.aqxpharma.com)."

There have been three published papers, two in vitro and animal models and one in vivo in humans characterizing AQX-1125. The first describes the effects of cell activation and chemotaxis in vitro (Stenton et al. 2013b). This paper documents that this compound is suitable for testing in various models of inflammation. The second paper shows the effects of AQX-1125 in rodent models of pulmonary and allergy. The efficacy of AQX-1125 is dependent on the presence of SHIP-1 (Stenton et al. 2013a). Finally, AQX-1125 was demonstrated efficacious in humans with mild to moderate asthma (Leaker et al. 2014). In this manuscript AQX-1125 significantly reduced the late response to allergen challenge with a trend in reduction of inflammation. Clinical side effects were very mild and did not lead to discontinuation of therapy. This was performed in a randomized, double-blind placebo-controlled, two-way crossover study in 22 steroid-naïve mild-to-moderate asthmatic individuals with documented late-phase response to inhaled allergen. These results might suggest a role for this SHIP-1 agonist in HRF/TCTP- induced symptoms.

16.9 Summary

In conclusion, further defining the extracellular role of the mechanism of HRF/TCTP-induced priming in vivo using our HRF/TCTP inducible-transgenic mouse and in vitro using both peripheral blood-derived basophils and CD34+ peripheral-derived cultured basophils could yield additional insight into HRF/TCTP's participation in the propagation of the Th2 asthmatic allergic response. The successful completion of these studies could lead to an inhibition of the function of this unique cytokine and its amelioration of its role in the allergic, asthmatic diathesis. SHIP-1 agonists may well be useful as therapeutic targets for the actions of HRF/TCTP in allergic responses.

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