A Novel Human Hormone-peptide NEROFE Selectively Induces Apoptosis in Cancer Cells through the T1/ST2 Receptor.

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Abstract: - We report the action mechanism of a novel Tumor-Cells Apoptosis Factor (Nerofe®). We find that Tumor-Cells Apoptosis Factor (TCApF) binds to the T1/ST2 receptor and activates caspases 8, 9 and 3 mediated apoptosis, together with activation of JNKinase and p38 MAPKinase. Application of TCApF to cells induced apoptosis in acute myeloid leukemia proliferating cells (U937 premeyloid cells), in human breast carcinoma (MCF7), human glioblastoma, human and murine neuroblastoma, human prostate cancer, murine mammary adenocarcinoma and human lung cancer proliferating cells. In contrast, TCApF was unable to induce apoptosis in non-proliferating cells. The selectivity of TCApF-induced apoptosis is related to the level of T1/ST2 receptor expression. This is the first report linking the T1/ST2 receptor to apoptosis.

Key-Words: - apoptosis, T1/ST2, cancer.

1 Introduction

T1/ST2 (IL1RL1) receptor is a member of the Toll/Interleukin-1 receptor (IL1R) superfamily. Originally, T1/ST2 was discovered in fibroblasts as an early response gene following over-expression of the v-mos or Ha-ras oncogenes or following serum activation [1,2]. It was also shown that LPS stimulates T1/ST2 expression in monocytes, muscle cells and splenocytes both in vitro and in vivo [3]. The T1/ST2 receptor is expressed in macrophages, dendritic cells and in mast cells as well. This receptor is a stable marker of Th2 polarized thymocytes (but not of Th1 polarized thymocytes) and is important in Th2's response to both viral antigen and allergens [4-6]. During the last few years it has been shown that T1/ST2 plays an important role in various diseases, in particular, cancer, inflammatory diseases, trauma, sepsis, cardiovascular diseases and idiopathic pulmonary fibrosis [4-6].

Until recently it was impossible to identify the T1/ST2 ligand. Monoclonal antibodies raised against T1/ST2 receptor were able to activate MAPKinase and JNK but not NF-kB. Lately a new member of the interleukin family, IL-33, was claimed to be a ligand for T1/ST2 receptor, but unlike monoclonal antibodies, IL-33 was shown to activate both MAP kinase and NF-kB [7].

The role of T1/ST2 in immuno modulation of the anti inflammatory response is the subject of many reports (4-9) but its role in cancer cells still remains an enigma. In spite of T1/ST2 being highly expressed in several human cancers such as breast carcinoma, NSCLC cells and AML cells [10], its role in these cells was unknown.

Screening the human genome with bioinformatical tools enabled us to find a chromosome sequence in, which encodes a protein with a signal peptide sequence at its N-terminus. In the mouse genome the analog sequence was found in chromosome 19. RT-PCR screening of human cDNA libraries prepared from different tissues enabled us to identify a new cDNA present mainly in the human thymus and partially in the colon and the frontal lobes of the brain. Predicted secondary structure of the peptide, its size, solubility and compactability imply that the peptide is most likely a hormone-like protein. The peptide without the signal sequence was named Tumor-Cells Apoptosis Factor (TCApF) [15].

Isolation of TCApF shed light on the role of the T1/ST2 receptor in cancer cells. In this paper we present data that describe the binding of TCApF to T1/ST2 receptor and how TCApF modulates the activity of T1/ST2 in cancer cells. We found that T1/ST2 is a selective death receptor for proliferating cancer cells and that it is naïve ligand of the receptor. Binding TCApF to T1/ST2 activates both caspases 8 and Bcl-2 mediated downstreams in cells that highly express the receptor. These

processes culminate in apoptosis of the cancerous cells.

2 Results

2.1 Interaction of TCApF with the T1/ST2 receptor

First, we tested whether TCApF can bind directly to T1/ST2 receptor. We used Fc-T1/ST2 recombinant protein (a protein composed of Fc and the extra cellular binding domain of the T1/ST2 receptor). Binding was shown by a co-precipitation assay in which biotinylated TCApF was used to bind Fc-T1/ST2. TCApF was subsequently, precipitated by protein A beads after addition of streptavidin in order to monitor the biotinylated TCApF (see Fig.1A). The Kd for the interaction of TCApF with T1/ST2 was calculated from a Scatchard plot to be 100 nM (Fig.2C). Recombinant Fc-ST2 protein was used in order to calculate the Kd of NF40 to ST2 receptor. We used HRP-avidin to calculate the amount of bound TCApF. As a control we used Fc-SIGGIR (a protein composed of Fc and the extra cellular binding domain of Toll/Interleukin-1 family member SIGGIR receptor) and we saw no binding. Non specific binding of TCApF to the plastic has been measured as well.

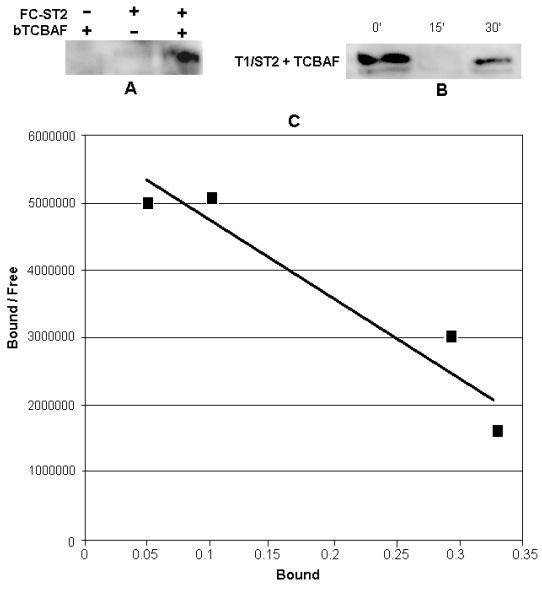

Fig.1. Interaction of TCApF with the T1/ST2 receptor.

A - Binding of biotinylated TCApF to recombinant Fc- ST2 was monitored by co-immunoprecipitation essay, with the complex being precipitated by portein-A beads and followed by streptavidin.

B - U937 cells were challenged with 100ng/ml of TCApF at time 0. Before addition of TCApF and at the times indicated thereafter, equal amounts of cells were essayed for the presence of T1/ST2 receptors by western blotting with anti-T1/ST2 monoclonal antibodies.

C. Scatchard plot of binding of TCApF to T1/ST2 receptor.

The interaction of TCApF with the T1/ST2 receptor was also documented based on pre-incubation of U937 cells with TCApF. The result is a subsequent disappearance of T1/ST2 receptors, most probably due to internalization of the TCApF-T1/ST2 receptor complex (Fig. 1B).

These results were confirmed in experiments with ST2−/− knockout mice (Fig.2). Splenocytes were isolated from wild type Balb/C males and from ST2−/− knockout Balb/C mice. The cells were incubated with LPS in order to increase ST2-receptor expression [3]. After that, the cells were treated with TCApF for 18 hours. As will be shown below (Sec.2.2), TCApF initiate apoptosis with DNA fragmentation in cells that highly express T1/ST2 receptors. Following the process mentioned above nuclear DNA was extracted from the splenocyte cells and analyzed on Agarose gel. Gel was stained with EtBr.

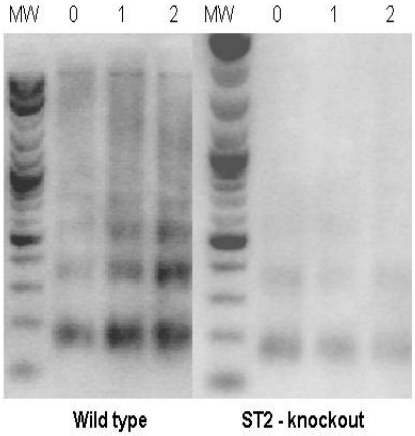

Fig. 2. DNA fragmentation in ST2−/− knockout and wild type mice splenocytes were treated with different concentrations of TCApF. MW molecular weight ladder, 0 – saline, 1 - 1ng/ml of TCApF, 2 - 10ng/ml of TCApF.

It is clearly seen that DNA extracted from the wild type mice cells, undergoes considerable fragmentation, while DNA from ST2 knockout cells does not.

2.2 TCApF initiates apoptosis in proliferating cancer cells

To elucidate the signal transduction pathway of TCApF we monitored the activation of major enzymes and proteins involved in signal transduction pathways associated with apoptosis, following exposure of U937 cells to TCApF. Application of TCApF to the cells results in phosphorylation of FADD, so the amount of non-phosphorylated FADD was reduced (Fig.3A, the antibodies against the non-phosphorylated FADD were used). Phosphorylation of FADD causes

degradation of full-length caspase 8 and full-length caspase 3 (Fig.3A).

The degraded caspases are the active species in the system and their activation is accompanied by degradation of PARP (Fig.3A), ICAD and DNA fragmentation (Fig.3C; 1, 2, 3).

The selectivity of the effect of TCApF is most probably due to the fact that the level of expression of T1/ST2 is highly dependent on the proliferative state of the cells.

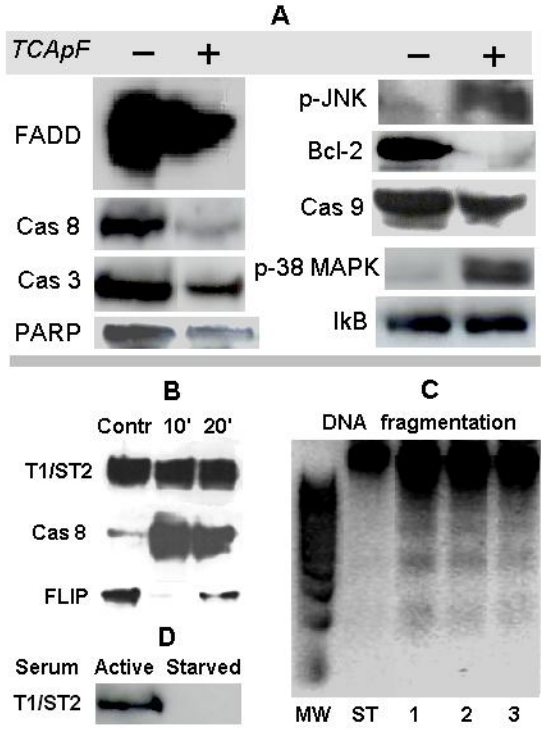

Fig. 3. TCApF induces apoptosis in proliferating cancer cells.

A - Equal amounts ($3x10^6$) of U937 cells were challenged with and without TCApF. Western blot of the indicated proteins was carried out. The antibodies used to detect caspase 8 and caspase 3 were against the full-length inactive forms of the caspases.

B - Time course of the effect of TCApF on the degradation of FLIP and activation of caspase 8. (It should be emphasized that unlike the results presented in Fig.3A, the antibodies against the active cleaved species of caspase 8 were used here).

C - DNA fragmentation. TCApF was applied to U937 cells. Nuclear DNA was extracted from cells after 24h and analyzed on Agarose gel. Gel was stained with EtBr. MW – molecular weight ladder**,** ST **–** non- proliferating (starved) cells are treated with TCApF. 1, 2 and 3 – decreasing concentration of TCApF has been applied to proliferating cells.

D - The effect of serum starvation on T1/ST2 expression. U937 cells were grown either in the presence of 10% serum (serum active), or 0.1% serum (serum starved). The amount of T1/ST2 was monitored with anti-T1/ST2 monoclonal antibodies.

Fig. 3D demonstrates that T1/ST2 is visible only serum-activated cells, whereas, it is invisible in starved cells.

In the next step we applied TCApF to U937 cells. At different time points we immuno-precipitated T1/ST2 from the cells and analyzed by western blot the presence of cleavage of the full-length caspase 8 and FLIP (Fig.3B). It is clear seen that in untreated cells a significant level of FLIP is observed. After treatment with TCApF the level of FLIP decreases and a concomitant increase of caspase 8 activation was observed (Fig.3B; 10',20'). The changes in FLIP and caspase 8 are time-sensitive, with the greatest changes seen within a short time.

Fig.3A shows that TCApF causes a very pronounced degradation of the anti-apoptotic protein Bcl-2, accompanied by cleavage of the full-length caspase 9. This, in turn, activates caspace 3.

Both caspase 8 and Bcl-2 mediated downstreams lead to fragmentation of DNA and this causes apoptosis of the proliferating cancer cells. Apoptosis was directly demonstrated by DNA fragmentation induced by TCApF addition to proliferating U937 cells (Fig.3C;1,2,3). DNA fragmentation was also found in human breast cancer cell line MCF7 and in lung cancer cells (not shown). Most importantly, serum-starved U937 cells in which proliferation was stopped, did not undergo apoptosis by TCApF application (Fig.3C;ST). Thus apoptosis was initiated only in the proliferating cancer cells.

Fig.3A also shows that TCApF causes an increase in both JNK and MAPK phosphorylation 30 min after TCApF application, while no change in IkB protein level was detected. No change of IkB level was detected at shorter time points as well. Therefore, the JNK pathway and MAP kinase pathway are activated by TCApF, while NF-kB remains deactivated.

2.3 TCApF downregulates angiogenic factors

Angiogenesis is one of the key processes in the development of tumors. It was interesting to investigate the effect of T1/ST2 receptor activation by TCApF on the expression of various components of the angiogenic mechanism.

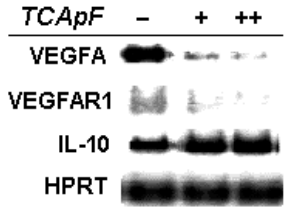

Fig.4. TCApF downregulates angiogenic factors expression. Mouse spleenocytes were challenged without TCApF or with increasing concentrations of it. VEGFA, VEGFR1, IL-10 expression were compared. HPRT was included as a negative control.

Indeed, it is seen in Fig.4 that TCApF inhibits the expression of the VEGFA and VEGFR1 receptor in mouse spleenocytes. VEGF is a major proangiogenic cytokine, VEGFR1 is the receptor for VEGF. On the other hand, in the same cells, TCApF enhanced the expression of IL-10, which is a known anti-angiogenic interleukin. Upregulation of angiogenic factors has been demonstrated in endothelial cells that are mediated by TLR4. Since T1/ST2 activation is known to counteract TLR4 activity, it is anticipated that TCApF would cause downregulation of angiogenic factors also in endothelial cells, in much the same way as it does so in immune system cells.

3 Discussion

TCApF (Nerofe®) is a compact and water soluble human hormone-like peptide, which has a mouse homologue. In all above mentioned experiments we used only a chemically synthesized peptide of purity 96%, which didn't contain the predicted signal peptide.

We found that TCApF can bind to T1/ST2 receptor. Pepride concentration of 10-100 ng/ml s activates FADD, capases 8 and 3, and p-JNK and p38 MAPK in proliferating pre–myeloid U937 cells.

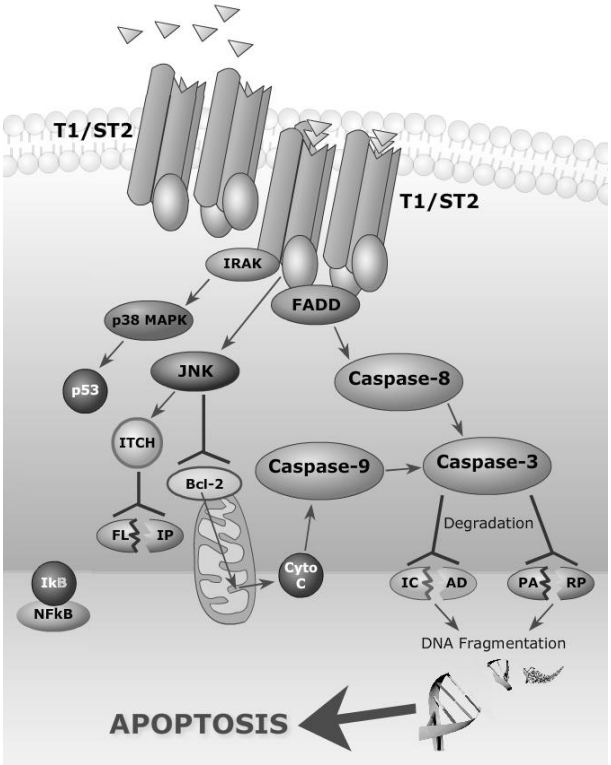

Fig.5. Apoptotic TCApF signaling pathways in the proliferating cancer cells.

We also observed degradation of Bcl-2, activation of capase 9, degradation of PARP and ICAD and DNA fragmentation (Fig.3). On the other hand IκB was not degraded, which implies that NF-κB was not activated.

It should be noted that these effects take place in proliferating cancer cells, while in the starved cells and healthy monocytes DNA fragmentation was not observed. The same results were obtained for human breast cancer cell line MCF7, human prostate cancer cell line PC3 and lung cancer cell line QG56 (not shown). All these results may suggest a T1/ST2+TCApF signaling pathway, which is shown in Fig.5. We also observed that proliferating U937 cells highly expressed T1/ST2 receptor, while in non-proliferating U937 cells (Fig.3C) and in healthy monocytes expression of the receptor is low (not shown).

In a previous reports, Brint *et al* [9,11] have investigated T1/ST2 signal transduction, using either transient over-expression of T1/ST2 or a cross-linking monoclonal antibody to activate receptors. They demonstrated that the anti-T1/ST2 antibody does not activate the transcription factor NF-kB in murine thymoma EL4 cells, or in mast cell line P815 that over-expressed T1/ST2. Anti-T1/ST2 antibody was, however, able to increase phosphorylation of c-Jun N-terminal kinase (JNK) and activate the MAP kinases, p42/p44 and p38. They also demonstrated that this effect was blocked by prior treatment with the JNK inhibitor SP600125.

Anti-T1/ST2 antibodies also induced the selective expression of IL-4 but not IFN-G in naive T cells.

Results in [9,11] are complementary to the described above, namely, JNK and MAPK activation with no change in IκB protein level.

It has been reported that JNK activation induces degradation of Bcl-2 protein [12] that leads to caspase 9 mediated apoptosis. Indeed, we observed that Bcl-2 is degraded following treatment with TCApF that was accompanied by a breakdown of the full-length caspase 9, denoting an activation of the protease. Hence, activation of T1/ST2 receptor by TCApF represents an interesting case where both the caspase 8 and Bcl-2 mediated apoptosis pathways are co-existing.

TCApF concentrations of 10-100 ng/ml were necessary to induce apoptosis in U937 cells, while lower concentrations in the range of 1-100 pg/ml did not induce apoptosis, although they did stimulate IL-4 and IL-10 synthesis in hPBL (not shown).

We suggest the following hypothetical mechanism for the selectivity of TCApF apoptotic activity exclusively towards proliferating cancer

cells. Over-expressing of T1/ST2 receptors in the cancer cells increases the probability for massive clustering of T1/ST2 receptors and, perhaps, aggregation of T1/ST2 with other receptors (an example of similar clustering for TNF-R receptor has been reported in [17]). In this case high concentrations (10-100 ng/ml) of TCApF activate the whole T1/ST2-cluster that leads to activation of FADD, which in turn activates caspase 8 mediated downstream that culminated in apoptosis.

In normal cells, however, expression of T1/ST2 is low, so the receptors operate, mainly, in a "single-receptor" mode. Binding of TCApF to a single receptor activates IL-4 and IL-10 synthesis and inhibits of IL-R1 and TLR4 receptors, but it does not activate FADD, since the intracellular domain of T1/ST2 is TIR-like and does not contain death domains. (A similar situation, apparently, takes place for low concentrations of TCApF (1-100 pg/ml) that cannot activate the whole TCApF-clusters even in the cells with over-expression of T1/ST2). Such a mechanism could explain the fact that low concentrations of TCApF are sufficient for the anti-inflammatory effects, whereas, high concentrations are needed for the apoptotic effects.

Selectivity of TCBAT functioning is reminiscent of the action of TRAIL [14], though there is a substantial difference: Quite often, activation by TRAIL is accompanied by increased levels of FLIP. FLIP activity inhibits caspase 8, thereby interrupting caspase 8 mediated apoptosis. In our case FLIP itself is inactivated. There might be 2 causes for this inactivation: 1. Inhibition of FLIP by ITCH which is activated by JNK [13]. 2. Since NFκB is not activated, synthesis of FLIP is halted [13]. In addition, TRAIL has no effect in Bcl-2 enriched cells, while TCApF almost completely degrades Bcl-2 (Fig.3A).

Materials and Method

Peptide synthesis and polyclonal antibodies

TCApF and its FITC derivative were synthesized by ANASPEC Inc, San Jose, CA 95131. The peptide used for the experiments was 96% pure by HPLC. Polyclonal antibodies were raised by ANASPEC Inc, San Jose, CA 95131.

Mice

Balb/C wild type mice were purchased from Harlan (Jerusalem, Israel). T1/ST2−/− knockout mice were as described in Townsend et.al. [16] and had been back crossed to the BALB/c background for 6 generations and were provided by Andrew N.J. McKenzie, (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom). All experiments were approved by the Institutional Animal Care and Use Committee.

Western blot analysis

$3x10^6$ U937 cells were grown in RPMI medium supplied with 10%FCS. The treatments were done over night. Cell were harvested and treated with Ripa buffer (Pierce Ltd supplemented with protienase and phosphatase inhibitors). Cell-lysis was subjected to SDS-PAGE analysis followed by western analysis using the following antibodies: anti PARP, anti ICAD, anti full length capase 3, anti full length caspase 9, anti full length and cleavage caspase 8, anti IκB, and Bcl-2 (Santa Cruz Biotech) anti phosphorylated p38 MAP kinase, anti phosphorylated p42/44 JNK, anti non-phosphorylated FADD and anti T1/ST2 (R&D systems).

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References

[1]. Tominaga S. A putative protein of a growth specific cDNA from BALB/c-3T3 cells is highly similar to the extracellular portion of mouse interleukin 1 receptor. *FEBS Lett 1989;* 258: 301-304.

[2]. Werenskiold AK, Hoffmann S, Klemenz R. Induction of a mitogen-responsive gene after expression of the Ha-ras oncogene in NIH 3T3 fibroblasts. *Mol Cell Biol 1989*; 9: 5207-5214.

[3]. Saccani S, Polentarutti, N., Penton-Rol, G., Sims, J.E., Mantovani, A. Divergent effects of LPS on expression of IL-1 receptor family members in mononuclear phagocytes in vitro and in vivo. *Cytokine 1998;* 10: 773-780.

[4]. Xu D. Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J Exp Med 1998;* 187: 787-794.

[5]. Meisel C, Bonhagen K, Lohning M, Coyle AJ, Gutierrez-Ramos J-C, Radbruch A, Kamradt T. Regulation and Function of T1/ST2 Expression on CD4+ T Cells: Induction of Type 2 Cytokine Production by T1/ST2 Cross-Linking. *J Immunol 2001;* 166: 3143-3150.

[6]. Coyle AJ. Crucial role of the interleukin 1 receptor family member ST2 in T helper cell type 2-mediated lung mucosal immune responses. *J Exp Med 1999;* 190: 895-902.

[7]. Schmitz J, Owyang, A., Oldham, E., Song, Y.,

Murphy, E., McClahahan, T.K., Zurawski, G., Moshrefi, M., Qin, J., Li, X., Gorman, DM, Bazan, F., Kastelein, RA IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity 2005;* 23: 479-490.

[8]. Sweet MJ, Leung BP, Kang D, Sogaard M, Schulz K, Trajkovic V, Campbell CC, Xu D, Liew FY. A Novel Pathway Regulating Lipopolysaccharide-Induced Shock by ST2/T1 Via Inhibition of Toll-Like Receptor 4 Expression. *J Immunol 2001;* 166: 6633-6639.

[9]. Brint EK, Xu D, Liu H, Dunne A, McKenzie ANJ, O'Neill LAJ, Liew FY. ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. *J Biol Chem 2004;* 5: 373-379.

[10] .Truong PT, Bernstein, V., Wai, E., Chua, B., Speers, C., Olivotto, IA Age-related variations in the use of axillary dissection: a survival analysis of 8038 women with T1-ST2 breast cancer. *Int J Radiat Oncol Biol Phys. 2002;* 54: 794-803.

[11]. Brint EK. Characterization of signaling pathways activated by the interleukin 1 (IL-1) receptor homologue ST2. A role for Jun N-terminal kinase in IL-4 induction. *J Biol Chem 2002;* 277: 49205-49211.

[12]. Srivastava RK, Mi, QS, Hardwick, J.M., Longo, D.L. Deletion of the loop region of Bcl-2 completely blocks paclitaxel-induced apoptosis. *Proc Natl Acad Sci U S A. 1999;* 96: 3775-3780.

[13]. Chang L, Kamata, H., Solinas, G., Luo, JL, Maeda, S., Venuprasad, K., Liu, YC and Karin, M. The E3 ubiquitin ligase itch couples JNK activation to TNFalpha-induced cell death by inducing c-FLIP(L) turnover. *Cell 2006;* 124: 601-613.

[14]. Gupta S. Molecular signaling in death receptor and mitochondrial pathways of apoptosis. Int J Oncol 2003; 22: 15-20.

[15]. Sandler U, Devary O, Briatbard O, and Devary Y. Nerofe – a novel human hormone-peptide with strong anti cancer activity. *This volume. 2010.*

[16]. Townsend, M.J., Fallon, P.G., Matthews, D.J., Jolin, H.E. and McKenzie, A.N.J. T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary Th2 responses. *J. Exp. Med. 2000;* 191: 1069-1075.

[17]. Gulbins E, Grassme H. Ceramide and cell death receptor clustering. *Biochim Biophys Acta. 2002*; 1585(2-3):139-145.