# A Novel Human Cytotoxic Cell Activation Factor with anti-Cancer Activity

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*Abstract:* - We report the isolation of a novel human Cytotoxic Cell Activation Factor (CCAcF). We found that the cDNA of this protein is expressed mainly in the human pancreas and kidney and partially in the placenta. CCAcF is also expressed in monocytes and CD8 cells, but only when these cells are activated, not when they are in a resting state, while rested CD19 lymphocytes do express CCAcF. We showed that application of CCAcF to acute myeloid leukemia cells (U937 premeyloid cells), human melanoma (CAG), human pancreatic cancer cells (Panc1) and human prostate cancer cells (PC3) inhibits the proliferation *in vitro*. In contrast, CCAcF doesn't affect healthy cells. Treatment of mice with induced pancreatic cancer eradicated the tumor in 60% of the treated mice, even when treatment began in a late stage of cancer development.

*Key-Words:* Cytotoxic cell activation, pancreatic cancer.

## 1 Introduction

Xcyte Therapies, which activate T-cells and induce them to kill their targets, are an important line of defense against cancer cells. Activated T-cells are called Cytotoxic T-Lymphocytes (CTL) and they kill target cells by forcing them to commit suicide. Two mechanisms have been suggested for the CTL induced death pathway: One is the ligand/Fas system and the other is the Perforin/Granzyme B system [1]. It should be noted that in addition to directly killing cancer cells, T-Lymphocytes produce several anti-tumor agents like TNF-α, INF-γ, IL-17 *etc.*, which are currently in use for treatment of various cancers [2-4].

This paper reports about a novel human Cytotoxic Cell Activation Factor (CCAcF). Application of CCAcF to human peripheral white blood cells *in vitro* increases secretion of IL17, TNF-α, INF-γ, perforin and granzyme B. On the other hand, *in vitro* application of CCAcF to acute myeloid leukemia cells (U937 premeyloid cells), human melanoma (CAG), human pancreatic cancer cells (Panc1, BxPc3) and human prostate cancer cells (PC3) inhibits their proliferation, but doesn't influence proliferation of the healthy cells.

Pancreatic adenocarcinoma remains one of the deadliest cancers in the world. It claims over 6500 lives per year in the U.K., over 40,000 in Europe, nearly 19,000 in Japan and over 30,000 in US. With a five-year survival rate of less than 4%, pancreatic cancer is the fifth leading cause of cancer related deaths.

Animal models show that CCAcF treatment of mice with induced pancreatic cancer eradicates tumors in 60% of the mice even in cases where treatment began at a late stage of tumor development.

## 2 Results

### 2.1 Cloning the CCAcF gene and synthesis of the gene's product

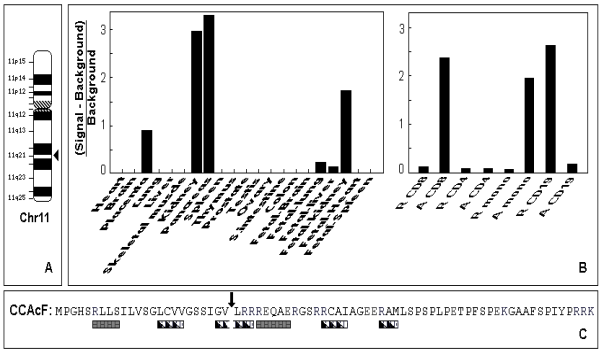


**Fig. 1. A** - localization of human CCAcF on chromosome 11 (black triangle). **B** - Equal amounts of cDNA from 30 different cDNA libraries (Heart, Brain, Placenta, Lung, Liver, Skeletal muscles, Kidney, Pancreas, Spleen, Thymus, Prostate, Testis, Ovary, S.intestine, Colon, Fetal-lung, Fetal-Brain, Fetal-liver, Fetal-kidney, Fetal-heart, Fetal-spleen, Fetal-Thymus. R CD19, R CD4, R CD8, R mono - cDNA libraries of the rested cells, A CD19, A CD4, A CD8, A mono - cDNA libraries of the active cells) were screened with specific oligonucleutides. As positive controls we used the GH gene and Total Genomic human DNA. **C** – Amino acid sequence of CCAcF. Black arrows show cleavage sites. Gray bars show alpha helixes and black-white bars show beta-strands.

Screening the human genome with bioinformatics

tools enabled us to find a sequence in chromosome 11/minus strand (HUM: 11q21; NT167190.11; GI: 224514855; region 40189268...40189047) [5], which appears to encode an open reading frame for a protein with a signal peptide sequence at its N-terminus (Fig.1). RT-PCR screening of human cDNA libraries prepared from different tissues enabled us to identify a new cDNA, which is mainly present in the human pancreas, kidney and partially in the placenta, but not in other tissues. The sequence of the RT-PCR product was identical to the predicted human sequence.

The new cDNA presents in several cells of the immune system, in particular, activated CTL and rested B-lymphocytes, but it doesn't present in rested CTL and activated B-lymphocytes. Note that new cDNA doesn't present in T-helper cells.

The human gene contains only one exon, which has initiation and termination codons and encodes a 74 amino acid protein (molecular weight 9.3333 kDa). Computational structural analysis of the deduced amino acid sequence revealed that the protein has a signal peptide at positions 1... 24. Predicted secondary structure of the peptide, its solubility and presence of the free peptide in human blood serum (Fig.2) imply that the protein is a secreted peptide.

We named the 50 amino acid peptide without the signal sequence Cytotoxic Cell Activation Factor (molecular weight 6.5371 kDa).

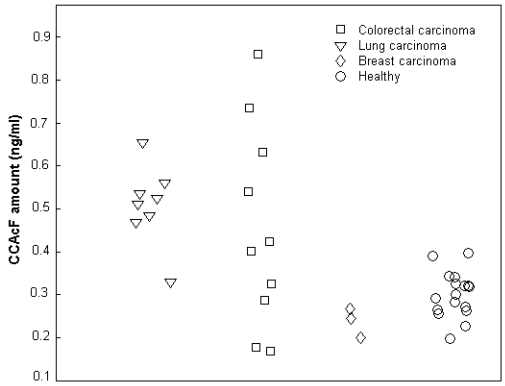


**Fig.2**. Amount of free CCAcF in samples of blood serum of the patients with different types of carcinoma and healthy persons.

## 2.2 CCAcF induces secretion of the cytokines that activate the CTL cells

We applied different concentrations of CCAcF to human peripheral white blood cells (pWBL) *in vitro* and observed a considerable increase of secretion of IL-17, TNF-α, INF-γ, perforin and granzyme-B (Fig.3). This observation indicates activation of the Cytotoxic T-Lymphocyte's response. This observation suggests that CCAcF is an activation factor of the CTL cells and could play an important role in defending our organism from developing cancer.

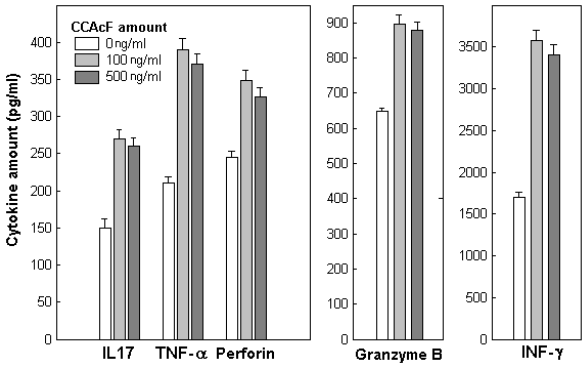


**Fig.3** Induction secretion of cytokines by human peripheral white blood cells *in vitro* after applying different concentrations of CCAcF. (Each experiment was repeated at least 3 times).

## 2.3 CCAcF inhibits proliferation of cancer cells *in vitro* and prevents tumor development *in vivo*, but doesn't affect healthy cells.

In order to check the anti-cancer activity of CCAcF, the peptide was incubated with different cancer cells for one day, and viable cells were detected using Resazurin and a spectrophotometer. The results are presented in Fig. 4.

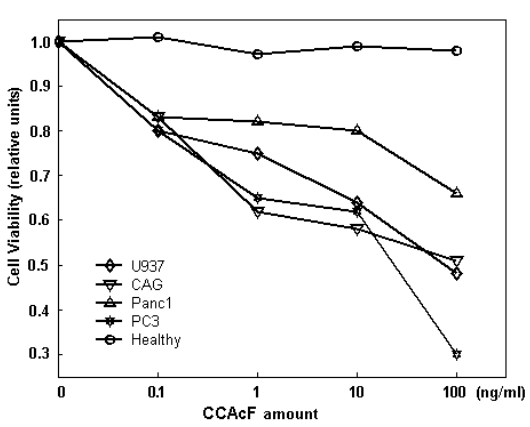


**Fig.4** CCAcF inhibits proliferation of the cancer cells in vitro. Equal amounts ($3\times10^5$) of the cells from different human cancer cell-lines and healthy cells were challenged with different concentrations of CCAcF during 24h (U937 – AML, CAG – melanoma cells, Panc1 – pancreatic carcinoma cells, PC3 – prostatic cancer cells, Healthy – healthy monocytes). Absorbance was measured after Resazurin application.

It can be seen that CCAcF causes a significant decrease in viable cells from these types of cancer cells, but doesn't influence proliferation of the healthy cells.

An animal model study correlated with our *in vitro* results. 8-week-old BALB/c nude mice were inoculated S.C. in the right flank (10 mice in each treatment group) with BxPc3 human pancreatic cancer cells. After 6 days the tumor became observable and the mice were randomly divided into 3 groups (each group containing 8 mice). Mice of the control group were treated by saline, while the treated mice groups were injected I.P. with 0.5mg/Kg and 1.25mg/Kg concentrations of CCAcF each every other day.

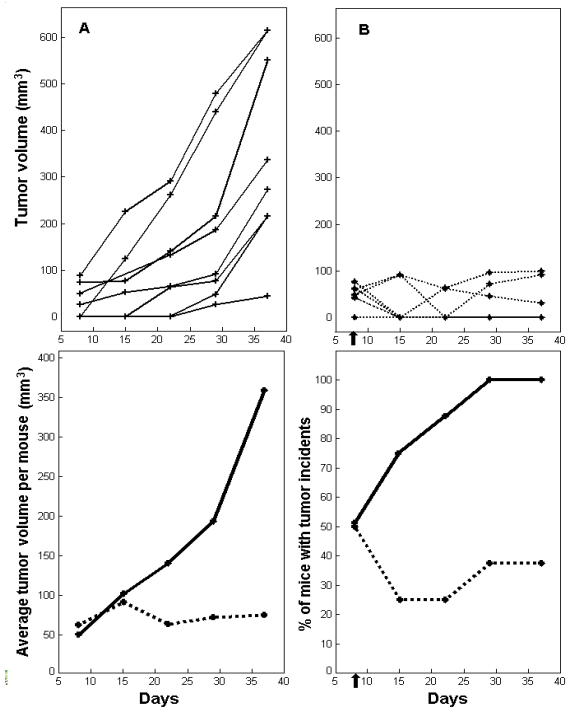


**Fig.5** Tumor growth in mice that have been injected with BxPc3 human pancreatic cancer cells. **A** - mice treated with saline. **B** - mice treated with 1.2mg/Kg CCAcF (Treatment began in an early stage of tumor development). **Solid lines** - mice treated with saline, **dashed lines** - mice treated with 1.25 mg/Kg CCAcF. Black arrows show beginning of treatment.

Tumor growth was monitored using calipers every 2 or 3 days. Fig.5 show the tumor size development for 30 days after the inoculations.

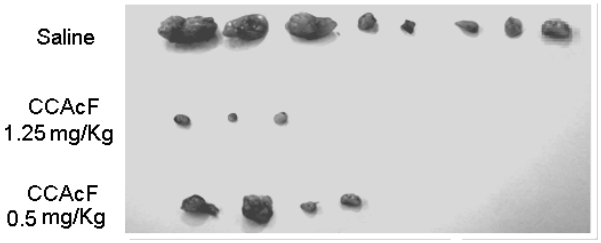


**Fig.6** Pancreatic tumor samples were extracted from 3 groups of mice, which were treated with different doses of CCAcF in an early stage of tumor development. Each group contains 8 mice. In the group treated with 1.25mg/Kg CCAkF only 3 mice slowly developed a tumor during 30 days, while in the other mice the tumor was eradicated.

It is clearly seen that both tumor size and cancer incidents were significantly lower in the 1.25mg/Kg CCAcF treated group in comparison with the control group. The tumor decreases in the dose dependent manner that is seen in Fig.6. It should be emphasized that CCAcF not only suppresses tumor growth, but also enables eradication of developed tumors.

In order to check whether CCAcF enables treatment not only of early stage tumor development, but also of later stage tumor development, we inoculated BALB/c nude mice with BxPc3 cells and waited until the tumor became well developed (100÷300 $mm^3$). After that the mice were randomly divided into 2 groups (each containing 7 mice). Mice in the control group were injected with saline, while mice in the treated group were injected I.P. with 1.25mg/Kg CCAcF 1×daily. The experiment was repeated twice with similar results.

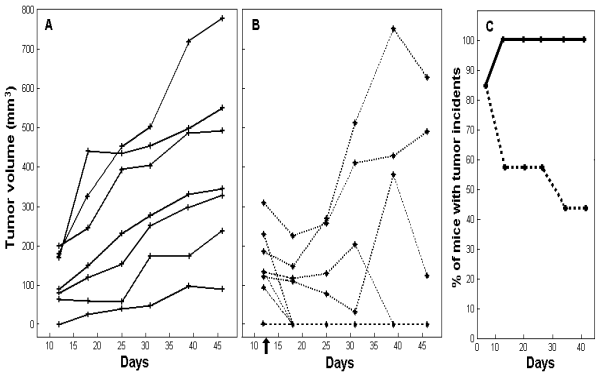


**Fig.7** Tumor growth in late stage of the tumor development in mice have been injected with BxPc3 human pancreatic cancer cells. **A** - mice treated with saline, **B** - mice treated with 1.25mg/Kg CCAcF. **Solid lines** - mice treated with saline, **dashed lines** - mice treated with 1.25 mg/Kg CCAcF. Black arrows show beginning of the treatment.

Table 1 (PathoVet Ltd.)

| Group | Dimension | Histological description (HPF: 5x40 fields) | Vessels /HPF | Mitosis /HPF | Hemorrhage |
|---|---|---|---|---|---|
| Control (Saline) | 4.5*3.5 mm | Lobulated mass with a larger solid component and high mitotic and angiogenesis indexes. | 4 | 9 | 1 |
| CCAcF (1.25 mg/Kg) | 4.0*3.1 mm | Larger ductal component (lesser solid proliferation) with low mitotic and angiogenesis indexes. | 1 | 1.4 | 0 |

The results (Fig.7) reveal that CCAcF enables tumor eradication in late stages of tumor development in

approximately 60% of the treated mice. It is important that in the other 40% of the mice in the treated group, most cancer cells were differentiated (non-invasive form), while the control group's tumor has a larger solid component (Table 1 and Fig.8).

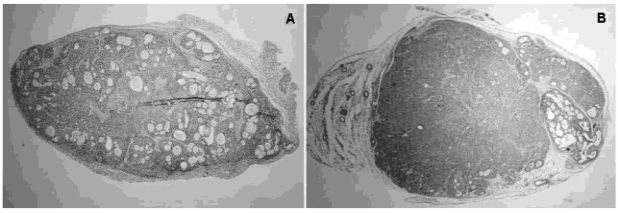


**Fig.8** Histology of the pancreatic tumor samples in a late stage of tumor development (PathoVet Ltd.). **A** - mouse treated with 1.25 mg/Kg CCAcF, **B** - control (mice treated with saline).

### 2.4 Toxicology study

A toxicology study was done by PathoVet Ltd. (81 Yehosa ben St., Rehovot 76391, Israel). Two groups of BALB/c mice (25 mice in each group) were injected with saline (control group) and with 6.0mg/Kg CCAcF (test group) for 28 days 1×daily I.P. There was no anemia in the smear. Polychromasia was evident. There was mild relative neutrophillia and lymphopenia. The neutrophils were mature without shift or toxicity and the monocytes were bland. No statistically significant difference in CBC and differential counts were found between control and test groups, except that the monocyte count in the test group was 2.5 times more.

## 3 Discussion

Pancreatic cancer presents one of the lowest 5-year survival rates and is a difficult case for chemotherapy [14]. Recently failed trials (e.g., Avastin from Genentech, USA Today, Jan. 24, 2007) point out the difficulty of bringing new drugs to market to treat pancreatic cancer**.** Gemzar is the current gold standard of care for unresectable and metastatic pancreatic cancer, but it is associated with overall survival of 5.7 months and a one-year survival rate of only 18%. It is therefore clear that treatment of pancreatic cancer continues to be plagued by high levels of unmet need.

We isolated and cloned the novel human secreted protein Cytotoxic Cell Activation Factor (CCAcF). We found that CCAcF is expressed mainly in the human pancreas and kidney, and partially in the placenta. It is also expressed in activated monocytes and CD8 cells, but not in these rested cells, while rested CD19 lymphocytes do express CCAcF.

Both *in vitro* and *in vivo* (mice animal model of pancreatic cancer) studies reveal strong anti-cancer activity of CCAcF. It is important that CCAcF was able not only to suppress pancreatic cancer development, but eradicate the tumor even in a late stage of tumor development.

Differentiation therapy is based on the concept that cancer cells are normal cells that have been arrested at an immature differentiated state, lack the ability to control their own growth, and so multiply at an abnormally fast rate [15-16]. Differentiation therapy aims to force the cancer cell to resume the process of maturation. Although differentiation therapy does not destroy the cancer cells, it restrains their growth and allows the application of more conventional therapies (such as chemotherapy) to eradicate the malignant cells.

A histological study (PathoVet Ltd.) shows that injecting BALB/c nude mice possessing well developed human pancreatic cancer with a 1.25mg/Kg dose of CCAcF enables transformation of cancer cells to a differentiated non-invasive form. This finding suggests that CCAcF could be used as a differentiation therapy agent.

A toxicology study of CCAcF treatment shows no considerable adverse effects at least up to a 5×(Therapeutic Effective Dose).

## Materials and Method

### Peptide synthesis and polyclonal antibodies

CCAcF was 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.

### RNA Harvesting and RT-PCR

RNA was harvested from cells by using Promega Kits (EZ RNA) and cDNA was prepared by using EZ cDNA kits (IBI Ltd). The following PCR conditions were used:
95˚C for 2 min. 40 cycles of : 95˚C for 45 sec, 59˚C for 45 sec, 72˚C for 5 min. End cycles: 72˚C for 5min. The product of the PCR was sequenced. Following the PCR analysis on Agarose gels and staining with Cybar Green (Invitrogene), the intensity of the PCR product was evaluated using BioRad ChemiDoc analyzer.

### Oligonucleotides

Oligonucleutides were synthesized by IDTDNA Ltd. The sequence of oligonucleutides we used for the screening of the different cDNA libraries were:
5'- CTGTGCGTTGTGGGTAGCAGCATTGGC-3'
5'- GCCCAGAAAAAGGAGCTGCTTTCTCCC-3'.

**Cell Lines**

Human pancreatic cancer cell lines Panc1 and BxPc3, human monocytic leukemia cell line U937, melanoma cell line CAG and prostate cancer cell line PC3 were grown in RPMI 1640 medium (Biological Industries, Kibbutz Beit Ha'emek, Israel) containing penicillin (100 units/ml), streptomycin (100 tzg/ml), amphotericin (0.25 /zg/ml), 10 /zM /3-mercaptoethanol, and 0.2% (w/v) glucose, together with 10% foetal calf serum (Biological Industries, Kibbutz Beit Ha'emek, Israel) (complete RPMI medium) in 5% $CO_2$/humidified air at 37°C, in suspension culture in 260 ml plastic bottles (Nunc, Denmark).

***In vitro* experiments**

U937 acute myeloid leukemia cells, CAG melanoma cells, Panc1 and BxPc3 pancreatic cancer cells and PC3 prostate cancer cells were each grown in 10% FCS + RPMI medium and quadruplicates were inoculated into a 96 well plate, 20,000 cells/well. Chemicals were applied to cells in different concentrations for 24 hours. Following this incubation Resazurin (R&D System) was applied to cells for 4 hours. Absorbance was measured by a spectrophotometer. Results represent quadroplicates. Each experiment has been repeated more than twice.

***In vivo* experiments**

BALB/c nude mice were purchased from Harlan (Jerusalem, Israel). Female nude mice were kept under a specific pathogen free (SPF) environment. All experiments were approved by the Institutional Animal Care and Use Committee. Mice were inoculated S.C. into the right flank of 8-week-old mice (10 mice for each treatment group) with $2\times10^6$ Panc1 or BxPc3 human pancreatic cancer cell lines in 0.2ml saline. Tumor growth was monitored using calipers every 2 or 3 days. Tumor volume was calculated as $(L*W^2)/2$, (where L = length and W = width) for 4 to 5 weeks. The control group was injected with saline, while the treated group was treated by I.P. injection of 100µl of different concentrations of CCAcF (1×daily for the period of the experiment).

**Blood test**

Samples of blood serum from patients with different types of cancer and blood serum of healthy persons were purchased from Asterand Partners in Human Tissue Research (SF 3807, USA). Samples were kept under -80ºC. Amount of native TCBAF in the blood was analyzed by standard Elisa procedure with CCAcF polyclonal antibody.

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*References*

[1]. Berke G. The CTL's kiss of death. *Cell*, 1995, 81:9–12.

[2]. Cun-Yu Wang, Marty W. Mayo, Albert S. Baldwin Jr., TNF- and Cancer Therapy-Induced Apoptosis: Potentiation by Inhibition of NF-kappa B *Science*, 1996, 274:784 - 787

[3] Eggermont A.M., Lienard D, Schraffordt Koops H. et al., Treatment of irresectable soft tissue sarcomas of the limbs by isolation perfusion with high dose TNF-alpha in combination with interferon-gamma and melphalan. *In: Fiers W, Buurman WA, eds. Tumor Necrosis Factor: Molecular and Cellular Biology and Clinical Relevance. Basel: Karger,* 1993: 239–243.

[4]. Eggermont A.M, Schraffordt Koops H, Lienard D et al. Isolated limb perfusion with high-dose tumor necrosis factor-alpha in combination with interferon-gamma and melphalan for non-resectable extremity soft tissue sarcomas: a multicenter trial. *J Clin Oncol*, 1996;14:2653–2665.

[5]. Sandler T. and Devary O., Novel protein, *PCT Int. Appl.* No: PCT/IL2008/001674 filed 25/12/08. Applicant: Two-To-Biotech Ltd. Publication: issued 2009/6.

[10]. Brint E.K, 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.

[11] Charles D. Surh and Jonathan Sprent, T-cell apoptosis detected in situ during positive and negative selection in the thymus, *Nature*, 2002, 372:100-103; Jose Alberola-Ila, Hogquest K. A., Swan K.A, Bevan M.J., Perlmutter R.M., Positive and Negative Selection Ivoke Distinct Signaling Pathways, *J.Exp.Med.*, 1996, 184:9-18.

[12] Rosenkranz M.A., Jackson D.C., Dalton K.M., Dolski I, Ryff C.D., Singer B.H., Muller D., Affective style and in vivo immune response: Neurobehavioral mechanisms, *PNAS*, 2003, 100:11148–11152 .

[13] R. Latagliata , V. Bongarzoni, I. Carmosino, A.

Mengarelli, M. Brecc, P. A. Borza , M. D'Andre , G. M. D 'Eli, S. Mecarocci, S. G. Morano, M. C. Petti, F. Mandelli and G. Alimena, Acute myelogenous leukemia in elderly patients not eligible for intensive chemotherapy: the dark side of the moon, *Annals of Oncology*, 2006, 17: 281–285.
[14]. Freelove R. and Walling A.D., Pancreatic Cancer: Diagnosis and Management, *American family Physician*, 2006, 73:485-92.
[15]. Beug1 H., Breast Cancer Stem Cells: Eradication by Differentiation Therapy?, *Cell*, 2009, 138:623-625.
[16]. Sell S., Cancer Stem Cells and Differentiation Therapy, *Tumor Biol*, 2006, 27:59-70.