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Cytokine Levels in Plasma and PHA Activated T Cells in Head and Neck Cancer Patients

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Abstract

Cytokines play a pivotal role in cancer, as these act as mediators in response to several physiological factors, caused by disease burden or interventions like surgery, chemotherapy and radiation. The levels of these cytokines can also act as biomarkers, and help in predicting a relapse episode or by analysing the efficacy of an intervention. Combination approaches based on the Cellular Immunotherapy are now being considered to reset the immune system thereby help achieve better prognosis in cancer treatment. One such approach is Autologous Activated T Cells Therapy (ATC), which entails *in vitro* activation of patient's own T cells from the peripheral blood and infusion of these activated T cells back into the patient. This helps in resetting the immune system which makes it conducive for better antigen presentation and a TH1 mediated anti-tumour response.

Since there is an immuno-suppressive milieu pre-existing in the body, it is important to check if the Autologous activation of T cells produces immunosuppressive cytokines, which would then undermine the intent of this approach.

In this study, we have measured the cytokines level in plasma and PHA stimulated mononuclear cells in control healthy population and head and neck cancer patients. Interleukin 10 (IL 10) and Transforming growth factor-beta (TGFβ) which fall under the anti-inflammatory category (pro-tumour) and Interferon-gamma (IFNγ) and Tumour necrosis factor-alpha (TNFα) which fall under the category of pro-inflammatory (anti-tumor) have been studied by ELISA.

Results show a significant decrease in plasma TGFβ levels in patients when compared to healthy controls. There is no increase in TGFβ levels on PHA stimulation, which is comparable to the healthy controls. There is an increase in plasma IL 10 levels in patients as compared to controls, indicating slight immunosuppression, although no significant increase in IL 10 is observed on PHA stimulation. There is a slight increase in plasma TNFα levels in patient population. On PHA stimulation, patient T cells produce significantly less TNFα when compared to control. There is a slight elevation in plasma IFNγ levels in patients, while on PHA stimulation; patient T cells produce comparable amounts of IFNγ, when compared to control.

These results indicate a) that Autologous Activated T Cell (ATC) does not result in amplification of TGFβ, and IL10 cytokines, b) Patients have the ability to produce IFNγ on PHA stimulation, indicating a functional immune system, c) PHA stimulation in patient results in less TNFα when compared to control, indicating a compromise in the T cell compartment.

Keywords: Cytokines; Head and Neck cancer; PHA; Activated T cells; Anti-inflammatory; Pro-inflammatory.

Introduction

Cytokines are small non-structural proteins which are released by cells and have a prominent role to play as mediators between cell to cell interactions and communication [1]. Cytokines are crucial in any immunological reaction as these proteins have a profound effect on the effector cells and also decide the fate of an immunological cascade [2]. Cytokines control differentiation, proliferation and survival of leukocytes and are important mediators in cancer. Cancer progression is deeply connected with the orchestra of cytokines in the body and their modulation by tumours *in vivo* [3]. Cytokines mainly fall under 2 categories: TH1 and TH2. CD4 helper T cells produce polarized TH1 and TH2 responses which evoke cell mediated immunity and strong antibody responses, respectively. [4]. TH1 cells produce IFNγ and TNFα amongst many others and are pro-inflammatory and anti-cancer by function whereas TH2 cells produce IL10 in addition to IL4, IL5, IL6, IL9, and are pro-tumorigenic, immuno-suppressive and anti-inflammatory. TGFβ is produced by many hematopoietic cell subtypes and is a pleiotropic growth factor which controls homeostasis, differentiation and peripheral tolerance in immune cells [5,6]. TGFβ is known to have diverse effects on cells based on its levels in blood. At higher concentrations they promote cancer whereas at lower concentrations show anticancer properties [7].Cytokine levels in cancer patients show higher TH2 cytokine patterns vis a vis TH1 profiles, thereby inducing a more immuno-suppressive milieu. This subverts the presentation of tumour antigens by dendritic cells, thereby

tolerizing these antigens and hindering the body's ability to mount an immune response. Treatment modalities to increase the efficiency of standard therapies could include cytokine infusions prior or during standard interventions thereby altering the immune status which could lead to a favourable outcome.

Combination approaches, like Activated T cells (ATC) are now being explored on the platform of Cellular Immunotherapy. This entails infusion of PHA activated T cells (after *in vitro* activation) into patients, which would modulate the immune suppressed environment and help the body fight the disease, resulting in optimal antigen presentation, furthered by reduced incidence of relapse. In patients, studies indicate an increased plasma TGFβ levels and TH2 type cytokine profile when compared to other TH1 type cytokines [7]. PHA stimulation of T cells could inadvertently activate these immunosuppressive cytokines, undermining the objective of Autologous ATC therapy. In this project, therefore we have evaluated the cytokine profiles of Head and Neck cancer patients in plasma and PHA activated T cell supernatants and compared them with corresponding healthy population, to objectively evaluate the merits of Autologous ATC therapy. Ethical clearances were obtained prior to the commencement of the study.

Patient derived PHA activation of T cells did not result in increase in TGFβ levels and IL 10 when compared to healthy control group, making autologous ATC a viable option for treatment under the ambit of Cellular Immunotherapy platform.

Materials and Methods

The population was selected under the framework of the research regulations of the Central Ethics Committee (CEC) of Health Care Global (HCG) Hospitals, Bangalore, India. For the study population to be selected, ethical clearance was obtained under reference number EC/363/17/09 and all the subjects whose blood samples were used for the study were given informed consent prior to their enrolment in the investigation.

A study group of 15 patients (30-60 years) diagnosed with Head and Neck cancer was selected for TGFβ, IL 10, IFNγ and TNFα level analysis. Additionally, a group of 25 healthy subjects were selected as a control group. It was ensured that all the healthy subjects fulfilled the criteria for blood donation, as set by the Blood Bank guidelines and were free of autoimmune and chronic diseases as well as infectious diseases during the course of the investigation. TGFβ, IL 10, IFNγ and TNFα cytokine levels were subsequently measured from the subjects' plasma as well as PHA stimulated T-cell supernatants, obtained from the blood samples.

Blood collection

15 ml of blood was collected from 25 healthy subjects via venous puncture, according to the standard Blood Bank collection procedure, and from the 15 Head and neck cancer patients during surgery, in EDTA vacutainers. The collected blood samples were used for plasma separation and mononuclear cell isolation for cytokine analysis.

Plasma separation and mononuclear cell isolation

The blood samples were centrifuged at 1500 rpm for 10 minutes, leaving the lighter plasma suspended above the denser cells. The plasma was isolated after centrifuging and stored at -800˚C until required. The remaining blood was diluted 3-fold with saline and mononuclear cells were isolated from the samples using ficoll-hypaque density gradient centrifugation for 20 minutes at 1500 rpm. The buffy coat was collected using a micropipette, washed with saline and counted. 2 million cells/ml were cultured in DMEM (Gibco, Invitrogen) with 10% bovine foetal serum and 100 ng/ml PHA (phytohaemagglutinin, Gibco) *in vitro* and incubated for 72 hours to allow T cell activation by PHA. After 72 hours, the T cell supernatant was collected and stored at -80˚C until required.

Cytokine level analysis

The levels of 4 cytokines, TGFβ, IL 10, IFNγ and TNFα were analysed by using Enzyme Linked Immuno Sorbent Assay (ELISA) kits, according to the manufacturer's (Ray BioTech) instructions. The concentration of these cytokines in the samples was determined by comparing them to standard curves for absorbance against concentration for each of the four cytokines.

Statistical analysis

The concentrations were expressed as mean values, with standard error mean used to express uncertainty in the mean values and bar graphs were constructed to visually depict the difference in cytokine levels. An unpaired Student's t-test was conducted between healthy volunteers and patients' cytokine levels in plasma and PHA stimulated T-cell supernatants and p value was obtained to determine the significance of the data using Graph Pad Prism software. p values<0.001 were considered to be significant.

Results

TGFβ, IL 10, IFNγ and TNFα levels were analysed in 15 Head and neck cancer patients and 25 healthy subjects were included in the study, with proper consents. The plasma levels and PHA stimulated T cell supernatants showed distinct patterns which was measured and analysed (Table 1).

Cytokines	Groups	Plasma levels (pg/µg protein)	PHA stimulated T cells supernatant (pg/µg protein)
TGFβ	Healthy	0.118 ± 0.005	0.046 ± 0.003
	Patients	0.075 ± 0.005	0.046 ± 0.001
IL 10	Healthy	0.00057 ± 0.00007	0.0034 ± 0.0002

	Patients	0.00078 ± 0.00008	0.0038 ± 0.0001
IFNγ	Healthy	0.006 ± 0.0003	0.07 ± 0.0094
	Patients	0.009 ± 0.0005	0.085 ± 0.0033
TNFα	Healthy	0.017 ± 0.0026	0.178 ± 0.0078
	Patients	0.024 ± 0.0015	0.106 ± 0.008

Table 1: Levels (pg/µg protein) of TGFβ, IL 10, IFNγ, TNFα in plasma and PHA stimulated T cells supernatant of healthy volunteers and head and neck cancer patients.

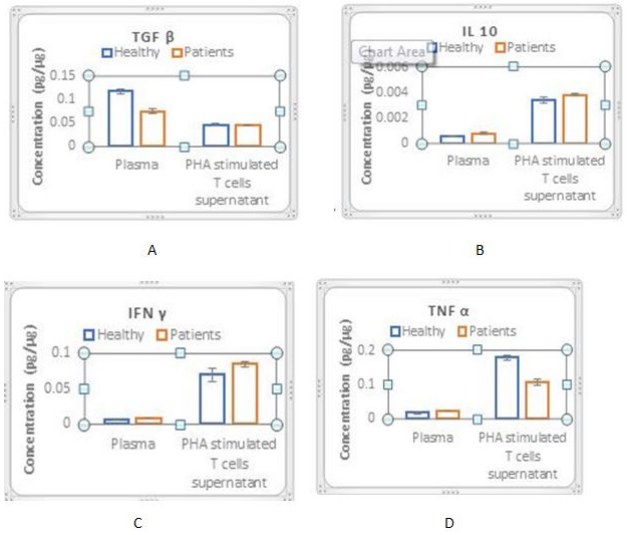

Figure 1: Graphical representation of (A) TGFβ (B) IL 10 (C) IFNγ and (D) TNFα concentration (pg/µg protein) in plasma and PHA stimulated T cells supernatant of both healthy volunteers and head and neck cancer patients.

Unlike IL 10, TGFβ levels in patients were significantly less ($p<0.001$) as compared to healthy subjects. On PHA stimulation, there was no increase in TGFβ and IL 10 levels indicating T cells in patients do not produce drastically elevated levels TGFβ (Figures 1A and 1B).

Although TNFα and IFNγ levels were slightly higher in healthy subjects, showed comparable levels of IFNγ after activation of T cells whereas a significant increase ($p<0.001$) in TNFα levels was observed in patients' ATC (Figures 1C and 1D).

Discussion

Head and Neck cancer arises in the oral cavity, pharynx, larynx, salivary glands and has the highest incidence in Indian population, which is also associated with high morbidity, metastasis and increased relapse rate. Cytokine profiles serve as biomarkers in such cases as tumour burden causes imbalances in the cytokine levels causing exacerbation of the disease and early relapse episode.

TGFβ is a pleiotropic cytokine with multifunctional effects on target cells. TGFβ signaling can induce anti-tumour as well as pro tumour response. TGFβ induces Epithelial to Mesenchymal Transition (EMT) and allows migration of suppressor cells like Macrophage Derived Suppressor Cells (MDSC) to migrate in the tumour microenvironment causing immune suppression.

When expressed and secreted, TGFβ is sequestered in the extracellular complex till it gets activated subsequently. Hence, there is a definite role of TGFβ in the tumour environment. TGFβ has been shown to be up-regulated in breast, colon, oesophageal, gastric, liver, lung, pancreas [8,9]. Serum concentrations in patients [10-20] have shown either increased levels [14,15,21] or decreased levels [22], indicating that there could be other factors contributing to changes in levels. Association of levels of TGFβ and stage of cancers have been explored, while some showing correlation [23,10,11] while others failing to show any correlation [12,13].

In a study in thyroid cancer patients [7], serum concentration levels of TGFβ were reported to be high in both healthy and patient population, although when compared with control, TGFβ levels measured less, although statistically not significant. This is similar to our findings indicating that cytokine levels in patients make a small contribution to the global cancer-immune interaction and the effects of TGFβ could be more profound in the tumour microenvironment. Moreover, decrease in TGFβ levels could indicate a loss of homeostasis, which could have far reaching consequences in a disease burdened person. Also, PHA stimulated T cells produced significantly less TGFβ when compared to healthy controls, which was not observed in our study, wherein no difference was reported. Lastly, as serum samples will have significant contribution of TGFβ from platelets, it is better to use plasma samples than serum samples [24-27]. This could be an important differential where high TGFβ serum levels are reported in other studies.

IL 10 is a TH2 type cytokine with immunosuppressive activity on immune cells. In our studies, we found increased levels of IL 10 in plasma levels of patients as compared to healthy subjects. Although no significant increase of IL 10 levels was found on PHA stimulation. Elevated IL 10 serum concentrations in Head and Neck cancer patients have been reported. It is elevated in stage III/IV than stage I/II of the disease and is an adverse prognostic factor [28]. Although not many reports have studied IL 10 levels in PHA stimulated T cells, this is interesting to note that there is no increase in IL 10 levels following PHA stimulation.

Levels of TNFα in plasma of patients were slightly elevated, PHA stimulation resulted in an increase in TNFα but this was lower than that in healthy subjects. This indicates a compromise in the T cell compartment in patients, although the role of TNFα as anti-tumourogenic is conflicting, as explained later in the paper.

In a similar study, conditioned media of head and neck cancer cell lines were tested on PBMCs, which on stimulation showed a significant increase in TNFα production just 6 hours post stimulation. Particularly, conditioned media from KB16 and HEP cells induced significantly increased levels of TNFα in PBMCs [29].

Currently the role of TNFα in Head and Neck cancers with respect to immune cells have not been fully elucidated [30-32]. TNFα has a paradoxical role to play in cancer. At high concentrations, TNFα has powerful anti-angiogenic and anti-cancer effects [33], however TNFα also has been shown to induce angiogenic factors resulting in tumour growth. TNFα is also shown to be involved in stroma modelling, induce DNA damage, and selection of resistant clones [32,34-36].

Our results on TNFα levels indicate a reduced ability of patients' T cells to produce TNFα, which could impinge the body's ability to mount an anti-tumour response, although considering the yin/yang nature of most of cytokines, including TNFα, more studies are required to conclude the findings.

Plasma IFNγ levels and the respective activated counterparts are comparable in both groups, indicating no difference in their respective levels. IFNγ is an integral TH1 cytokine and is attributed for its potent anti-tumoral activity. IFNγ signaling induces cell cycle arrest in tumour cells, inhibits angiogenesis, activating antigen presentation, and inhibiting migration of tumour suppressive cells in the tumour environment [37,38].

Conclusion

Our results thus indicate that the patient population under study are not compromised in their ability to produce IFNγ, but also indicates other factors could contribute to immune suppression. Autologous ATC therapy could be beneficial to such patients as their T cells retain their ability to produce IFNγ and hence render anti-tumour benefits.

Results obtained indicate that Autologous T cells (ATC) therapy can be considered as a viable treatment option as IL 10 and TGFβ are not amplified upon PHA stimulation when compared to healthy controls. These findings resonate with our earlier results wherein reference levels in healthy subjects showed no increased production of TGFβ.

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