

Research Article

About the Role of Insulin in the Interaction Between Human Immune and Colon Cancer Cells

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Abstract

Background: Insulin has been one of the immense contributions to human health. Besides its role in treatment of diabetes, insulin affects inflammatory cytokine production, chronic inflammation and cancer development. We have examined the effect of insulin on cytokine production by human peripheral blood mononuclear cells (PBMC) and its role in the balance between immune and human colon cancer cells.

Methods: Human LPS stimulated and non-stimulated PBMC, HT-29 and RKO cancer cell lines were separately incubated with insulin at various concentrations and the production of TNF α , IL-1 β , IL-6, IFN γ , IL-1ra and IL-10 was examined. In addition, the effect of insulin on the secretion of these cytokines by PBMC co-incubated with carcinoma cells was evaluated.

Results: Insulin added to non-stimulated PBMC caused a decreased secretion of the pro-inflammatory TNF α . Stimulation of PBMC with either LPS or PMA resulted in decreased production of IL-1 β , TNF α and IFN γ and an increased generation of IL-6. The production of the anti-inflammatory cytokine IL-10 was elevated by both stimulated and unstimulated PBMC. Insulin added to PBMC inhibited the secretion of IFN γ stimulated with both HT-29 and RKO cells and reduced TNF α production induced by RKO cells.

Conclusions: The results indicate that insulin interferes with inflammatory cytokine production by stimulated PBMC and it affects the cross talk between immune and colon cancer cells. It appears that the effect of insulin on

the immune balance between mononuclear and malignant cells depends on its concentration and type of cancer cells. The findings provide an additional way for understanding the relation between insulin and cancerogenesis.

Keywords: Insulin; Mononuclears; Cytokines; Colon carcinoma cells

Abbreviations

PBMC –Peripheral Blood Mononuclear Cells

IL-Interleukin

TNF –Tumor Necrosis Factor

IFN-Interferon

PBS-Phosphate Buffered Saline

FBS-Fetal Bovine Serum

CM- Complete Medium

MEM-Modified Eagle Medium

W/V-Weight/Volume

CS-Compound Symmetry

T2D-type two diabetes

1. Introduction

The narrative of insulin, the life-saving hormone that rescued the life of innumerable diabetic patients, is amazing since it combines knowledge, perception, and perseverance. The description of the apparently first cases of diabetes mellitus diagnosed centuries ago, the role of the pancreas in its etiology and the crucial phases of insulin elaboration have been reviewed by Quianzon and Cheikh [1]. In addition to its principal function as a regulator of glucose metabolism, insulin is closely linked with activity of myocytes, hepatocytes and adipocytes [2]. Studies have shown that insulin resistance observed in type two diabetes (T2D) is associated with the immune system. In a group of 45 insulin treated T2D patients the levels of the pro-inflammatory cytokines IL-2, IFN γ and IL-4 were found to be lower than those in non-treated T2D controls, whereas that of the anti-inflammatory IL-10 was significantly higher [3]. It has been suggested that insulin resistance observed in obese patients with T2D may be due to chronic inflammation related to the ability of immune cells, specifically M1 pro-inflammatory macrophages, to infiltrate the affected organs and overproduce inflammatory cytokines such as TNF α , IL-6, and IL-1 β [4,5]. Consequently, long-lasting inflammation results in a failure of pancreatic β -cell function, cell apoptosis and development of insulin resistance [6,7]. Furthermore, it has been established that persistent chronic inflammation is a key player in the process of cancer development [8, 9].

Macrophages, particularly the tumor associated ones, hold a pivotal role in carcinogenesis, in part by their ability to produce inflammatory cytokines. It appears that macrophages and insulin are closely related. Cultured monocytes are able to differentiate, synthesize and even secrete insulin [10]. It has been reported that macrophages engaged in apoptosis promoted by inflammatory-associated cytokines may produce insulin-like growth factor which in turn

binds to its receptor located on non-professional phagocytes and thus redirect their engulfing capacity [11]. These observations point to the existence of a functional chain between insulin, macrophages and cancer cells expressed by development of chronic inflammation, insulin resistance and carcinogenesis. In a previous study in our laboratory, we have demonstrated the existence of an immune dialogue between human peripheral blood mononuclear cells (PBMC) and colon cancer cells from two human lines [12]. It has been shown that this dialogue is modulated by a variety of factors impelling cytokine secretion by PBMC in the presence of cancer cells [13].

The present study encompassed a dual purpose i.e. to examine the effect of insulin on cytokine production by human PBMC and to detect if insulin may affect the immune balance between PBMC and HT-29 and RKO human colon carcinoma cells.

2. Materials and Methods

2.1 Cell preparation

2.1.1 PBMC

Peripheral blood mononuclear cells (PBMC) were separated from venous blood obtained from adult blood donors by Lymphoprep-1077 (Axis-Shield PoC AS, Oslo, Norway) gradient centrifugation. The donors gave written agreement and informed consent that components of their blood might be used for medical research. The cells were washed twice in phosphate buffered saline (PBS) and suspended in RPMI-1640 medium (Biological Industries, Beith Haemek, Israel) containing 1% penicillin, streptomycin and nystatin, 10% fetal bovine serum (FBS), and was designated as complete medium (CM).

2.1.2 Colon cancer cell lines

HT-29 and RKO human colon cancer cell lines were obtained from American Type Cultural Collection, Rockville, MD. The cells were maintained in CM containing Mc-Coy's 5A medium (Sigma, Israel) and Dulbecco modified eagle medium (DMEM- Biological Industries Co, Beth-Haemek, Israel) respectively, supplemented with 10% foetal bovine serum (FBS), 2mM L-glutamine and antibiotics (penicillin, streptomycin and nystatin-Biological Industries Co, Beth-Haemek, Israel). The cells were grown in T-75 culture flasks at 37°C in a humidified atmosphere containing 5% CO₂.

2.1.3 Insulin preparation

Insulin-R (Humulin regular, rDNA origin, human, Eli Lilly and Company, Indianapolis, USA) 100 units/ml (1 unit= 0.0347 mg) was used. It was diluted in CM and added at a final volume of 10 µl/ml. Control culture contained 10µl/ml of CM.

2.1.4 Effect of insulin on cell proliferation

The effect of insulin on PBMC and colon cancer cells proliferation was determined using XTT proliferation assay kit (Biological Industries, Beith Haemek, Israel). Briefly, 0.1 ml aliquots of PBMC or colon cancer cells (10⁵/ml of CM) were added to each one of 96 well plates and incubated for 24 hrs in the absence or presence of insulin at

concentrations as indicated. At the end of the incubation period, the cells were stained according to the manufacturer's instructions. The plates were incubated for 2-4 hrs at 37°C in a humidified incubator containing 5% CO₂, and the absorbance was measured at 450 nm using ELISA reader.

2.1.5 Effect of insulin on cytokine production

1.0 ml of PBMC (2×10^6 /ml of CM) was incubated without (non-stimulated) or with LPS (50ng/ml) for TNF α , IL-1 β , IL-6, IL-10 and IL-1ra production, or with 1 μ g/ml PMA and 0.5 μ g/ml ionomycin for IFN γ secretion. In another set of experiments, 0.5 ml of PBMC (4×10^6 /ml of CM) was incubated with 0.5 ml of either HT-29 or RKO colon cancer cells (4×10^5 /ml) suspended in proper CM.

Insulin was added at the onset of cultures at concentrations of 10^{-10} to 10^{-6} M. Cultures without insulin served as controls. The cultures were maintained for 24 hrs at 37°C in a humidified atmosphere containing 5% CO₂. At the end of the incubation period the cells were removed by centrifugation at 250 g for 10 min, the supernatants were collected and kept at -70°C until assayed for cytokines content.

2.1.6 Cytokine content in the supernatants

The concentration of TNF α , IL-1 β , IL-6, IFN γ , IL-10 and IL-1ra in the supernatants was tested using ELISA kits specific for these cytokines (Biosource International, Camarillo, CA) as detailed in the guide-line provided by the manufacturer. The detection levels of these kits were 15 pg/ml for IL-6, and 30 pg/ml for the remaining ones.

2.1.7 Statistics

A linear mixed model with repeated measures and the assumption of compound symmetry (CS) was used to assess the effect of different concentrations of insulin on cytokine secretion by non-stimulated or stimulated PBMC. SAS vs 9.4 was used for this analysis. Paired t-test was applied to compare between the level of cytokines produced by various concentrations of insulin and that found in control cultures. Probability values of $p < 0.05$ were considered as significant. The results are expressed as mean \pm SEM.

3. Results

3.1 Effect of insulin on cell proliferation

Insulin, added to the cells at concentrations between 10^{-10} M and 10^{-6} M for 24 hrs had no effect on PBMC, HT-29 or RKO cell proliferation rate as measured by XTT test.

3.2 Effect of insulin on inflammatory cytokine production

The secretion of IL-1 β , IL-6, IL-1ra and IFN γ by non-stimulated PBMC was not affected by 24 hrs of incubation with insulin at concentrations between 10^{-10} M and 10^{-6} M as compared with that produced by cells incubated without insulin for the same period of time. However, the production of TNF α by non-stimulated PBMC was slightly reduced and that of IL-10 was significantly enhanced following 24 hrs of incubation with the same insulin

concentrations (Tables 1-3). At insulin concentrations of 10^{-9} M and 10^{-8} M the secretion of TNF α by non-stimulated PBMC was lowered by 18.5% and 20% (Table 1), whereas IL-10 production was elevated by 21% at insulin concentration of 10^{-10} M (Table 3). LPS treated PBMC incubated with insulin did not show any significant alteration of IL-1 β , IL-1ra and IL-10 secretion, whereas that of IL-6 by being enhanced (Tables 1,3). LPS-induced TNF α and PMA-induced IFN γ secretion was significantly reduced by insulin (Tables 1-2). At concentrations between 10^{-10} M and 10^{-7} M, the secretion of TNF α was reduced by 36%-11% and that of IFN γ by 9%-21%. LPS-induced IL-6 production was higher by 25%-16% at insulin concentrations of 10^{-10} M and 10^{-8} M.

3.3 Effect of insulin on HT-29 and RKO-induced cytokine production

Insulin added at concentrations between 10^{-10} M and 10^{-6} M had no effect on the secretion of TNF α , IL-1 β , IL-6, IL-1ra and IL-10 by PBMC co-cultured with HT-29 colon cancer cells and did not affect IL-6, IL-10 and IL-1ra secretion when induced by RKO cells. At the same concentrations of insulin, TNF α production by PBMC co-cultured with RKO cells was inhibited by 10%-30% (Table 1). The secretion of IFN γ induced by cancer cells of both colon lines was reduced by 43%-17% by insulin added at concentrations between 10^{-10} M and 10^{-8} M ($p < 0.05$) (Table 2).

Insulin, M	Non-stimulated	LPS-stimulated	HT-29	RKO
TNFα, pg/ml				
0	157 \pm 17	564 \pm 96	937 \pm 90	952 \pm 153
10^{-10} M	148 \pm 20	363 \pm 58**	799 \pm 69	652 \pm 70**
10^{-9} M	128 \pm 13†	465 \pm 82**	930 \pm 129	774 \pm 103†
10^{-8} M	125 \pm 14*	484 \pm 90*	946 \pm 145	833 \pm 109
10^{-7} M	132 \pm 20	503 \pm 91*	949 \pm 186	861 \pm 138
10^{-6} M	132 \pm 20	531 \pm 90*	958 \pm 162	844 \pm 126
Repeated measures	F _{5,29} =2.48, P=0.055	F _{5,29} =7.86, P<0.001	F _{5,25} =1.02, P=0.43	F _{5,25} =3.84, P=0.010
IL-1β, ng/ml				
0	0.36 \pm 0.01	5.57 \pm 0.82	5.07 \pm 0.70	4.99 \pm 0.60
10^{-10} M	0.43 \pm 0.04	5.31 \pm 0.93	4.52 \pm 0.67	3.35 \pm 0.64
10^{-9} M	0.34 \pm 0.02	5.17 \pm 0.91	4.79 \pm 0.77	3.50 \pm 0.76
10^{-8} M	0.31 \pm 0.02	5.28 \pm 0.86	4.53 \pm 0.70	3.65 \pm 0.73
10^{-7} M	0.30 \pm 0.02	5.05 \pm 0.75	4.82 \pm 0.75	3.73 \pm 0.66
10^{-6} M	0.31 \pm 0.02	4.80 \pm 0.78	4.62 \pm 0.74	3.54 \pm 0.64
Repeated measures	F _{5,25} =1.53, P=0.21	F _{5,25} =2.01, P=0.11	F _{5,25} =1.7, P=0.17	F _{5,25} =1.42, P=0.25
IL-6, ng/ml				
0	1.30 \pm 0.24	16.12 \pm 2.18	15.3 \pm 1.24	13.47 \pm 2.36
10^{-10} M	1.49 \pm 0.26	20.16 \pm 2.76***	16.03 \pm 2.84	12.15 \pm 2.70

10⁻⁹ M	1.22±0.18	18.79±2.48*	12.99±4.10	12.78±3.40
10⁻⁸ M	1.38±0.29	18.23±2.15†	13.04±2.06	12.44±3.28
10⁻⁷ M	1.06±0.20	16.58±1.1	15.64±2.88	13.45±3.21
10⁻⁶ M	1.30±0.45	16.78±1.78	14.96±2.53	12.24±2.40
Repeated measures	F _{5,30} =0.47, P=0.79	F _{5,30} =5.63, P<0.001	F _{5,15} =1.13, P=0.38	F _{5,15} =0.38, P=0.85

Table 1: Effect of insulin on proinflammatory cytokine production by PBMC

Non-stimulated PBMC, or cells stimulated with LPS, HT-29, RKO were incubated for 24 hrs without (0) or with insulin at concentrations as indicated. The level of cytokines in the supernatants was tested by ELISA. The results are expressed as Mean ± SEM of 5-7 experiments. Asterisks represent statistically significant difference from cells incubated without insulin (*p<0.05, **p<0.01, ***p<0.001, †p=0.06).

IFNγ, ng/ml				
Insulin	Non-stimulated	PMA-stimulated	HT-29	RKO
0	0.61±0.04	24.7±4.1	1.36±0.27	3.98±1.20
10 ⁻¹⁰ M	0.59±0.04	22.5±3.7	0.87±0.16*	2.85±0.83**
10 ⁻⁹ M	0.59±0.04	21.2±3.7*	1.13±0.30	3.33±1.06*
10 ⁻⁸ M	0.59±0.02	21.3±3.5*	1.12±0.26	3.40±1.06*
10 ⁻⁷ M	0.64±0.04	19.6±3.5***	1.38±0.43	3.35±0.97
10 ⁻⁶ M	0.62±0.03	22.8±4.1	1.25±0.30	3.51±1.35
Repeated measures	F _{5,15} =2.47, P=0.08	F _{5,30} =5.38, P=0.0012	F _{5,25} =2.4, P=0.065	F _{5,25} =2.33, P=0.072

Table 2: Effect of insulin on IFN γ production by PBMC

Non-stimulated PBMC or cells stimulated with either PMA/ionomycin, or one of the colon cancer cell lines HT-29 or RKO, were incubated for 24 hrs without (0) or with insulin at concentrations as indicated. The level of cytokines in the supernatants was tested by ELISA. The results are expressed as Mean ± SEM of 5-7 experiments. Asterisks represent statistically significant difference from cells incubated without insulin (*p<0.05, **p<0.01, ***p<0.001).

IL-10, ng/ml				
Insulin, M	Non-stimulated	LPS-stimulated	HT-29	RKO
0	0.19±0.01	1.95±0.40	0.76±0.23	0.66±0.19
10 ⁻¹⁰ M	0.23±0.02**	2.11±0.43	0.70±0.20	0.72±0.26
10 ⁻⁹ M	0.20±0.01	2.27±0.52	0.73±0.22	0.68±0.20
10 ⁻⁸ M	0.20±0.02	1.82±0.30	0.68±0.21	0.65±0.20
10 ⁻⁷ M	0.18±0.01	1.97±0.35	0.76±0.24	0.76±0.26
10 ⁻⁶ M	0.17±0.01	1.82±0.33	0.70±0.23	0.64±0.19
Repeated measures	F _{5,29} =5.14, P=0.0017	F _{5,29} =2.51, P=0.052	F _{5,15} =0.99, P=0.46	F _{5,15} =1.05, P=0.43
IL-1ra, ng/ml				
0	1.20±0.12	2.29±0.14	1.62±0.17	1.60±0.18
10 ⁻¹⁰ M	1.13±0.11	2.20±0.15	1.59±0.22	1.58±0.22
10 ⁻⁹ M	1.14±0.23	2.25±0.15	1.62±0.20	1.64±0.24
10 ⁻⁸ M	1.12±0.20	2.31±0.10	1.48±0.23	1.69±0.25
10 ⁻⁷ M	1.17±0.22	2.38±0.08	1.58±0.21	1.71±0.28
10 ⁻⁶ M	1.13±0.22	2.20±0.10	1.68±0.24	1.63±0.21
Repeated measures	F _{5,15} =1.02, P=0.44	F _{5,15} =3.91, P=0.018	F _{5,25} =1.06, P=0.40	F _{5,25} =0.52, P=0.75

Table 3: Effect of insulin on anti-inflammatory cytokine production by PBMC

Non-stimulated PBMC or cells stimulated with either LPS, or with one of the colon cancer cell lines HT-29 or RKO, were incubated for 24 hrs without (0) or with insulin at concentrations as indicated. The level of cytokines in the supernatants was tested by ELISA. The results are expressed as Mean ± SEM of 5-7 experiments. Asterisks represent statistically significant difference from cells incubated without insulin (**p<0.01).

4. Discussion

The results of the current work indicate that insulin did not affect the secretion of the examined cytokines by non-stimulated PBMC at all concentrations applied, except for the pro-inflammatory TNF α generation that was inhibited at higher insulin concentration and the anti-inflammatory IL-10 its production being stimulated at the lowest concentration only. The results varied when cytokine production by PBMC was assessed following their stimulation either by LPS or PMA depending on the competency of immune cells to produce cytokines. The secretion of the proinflammatory cytokines TNF α and IFN γ was inhibited, whereas that of IL-6 was elevated. As for the anti-inflammatory cytokine IL-10, there was increased production with a concentration of 10⁻⁹M of insulin. The close

link between insulin and PBMC comprising monocytes, macrophages as well as lymphocytes, has been previously reviewed and their role in the development of T2D was highlighted [3,14,15]. PBMC are involved in the development of the so-called immune cell-induced insulin-dependent diabetes mellitus since they invade the pancreas, secrete pro-inflammatory cytokines - mainly IL-1 β , TNF α and IFN γ with a consequent apoptosis and destruction of the pancreatic β cells [16]. Iida et al. [17] reported that high concentration of insulin (10^{-7} M), but still in physiological limits, stimulates the gene expression of TNF α in macrophages through an extracellular-regulated kinase dependent pathway.

The possible interaction between PBMC and the two types of colon cancer cells used in the study under the impact of insulin is intriguing. Considering the fact that insulin may interfere with inflammatory cytokine production, the question was posed if addition of insulin to co-cultures of PBMC and colon cancer cells from the two lines used in the study will change the balance between immune and carcinoma cells. At that setting, insulin, mainly at lower doses, caused a decreased production of IFN γ – slightly expressed by HT-29 and considerably by RKO cells and reduces TNF α secretion prompted by RKO cells. The cancer cell type and existence of insulin dependency in this chain of events may serve as a possible explanation for this phenomenon. Studies have revealed a relationship between raised insulin level and the risk of colon cancer. In view of the fact that insulin, at least in vitro, acts as a mitogen [18] and patients with T2D and insulin resistance are more predisposed to cancer morbidity compared to healthy individuals [19,20], this assumption seems to be plausible.

In a survey comprising 9,778 hyperinsulinemic, but otherwise healthy subjects without diabetes, cancer mortality was significantly higher compared to those with normal blood insulin level [21]. Similar observations linking increased fasting insulin level with cancer death have been reported in other studies [22]. In recent publications Wintrob et al. [23,24] have shown that exogenous insulin prompts TH1 and TH2 helper cells to produce pro-inflammatory cytokines and activates tumor associated macrophages. Accordingly, maintaining chronic inflammation supplies a suitable milieu for cancer development. Working with HCT-116 human colorectal cells Lu et al. [25] have observed that insulin induced an increased cell proliferation and migration ability supporting the link between diabetes and cancer development. In the present study insulin did not affect HT-29 or RKO cells' proliferation rate.

The results of the current work show that insulin modulates inflammatory cytokine production by non-stimulated and mitogen stimulated PBMC. Furthermore, it interferes with the immune balance between PBMC and human colon cancer cells expressed by inhibition of IFN γ and TNF α production. The fact that malignant cells are capable to induce immune response by PBMC has been detailed in other studies [12] and has been designated as immune surveillance [26]. The present findings support this conception. The inhibited generation of IFN γ by PBMC stimulated for cytokine production by HT-29 and RKO cells and that of TNF α by RKO cells only in the presence of lower concentrations of insulin indicates the existence of a dose- and cell dependent effects and enlightens the role of insulin in the development of cancer.

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Conflicts of interest

None

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