The Task of Desferrioxamine and Iron in the Immune Interplay between Mononuclears and Colon Cancer Cells

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Received Date: December 19, 2016
Accepted Date: January 10, 2017
Published Date: January 16, 2017

Abstract

Background: Macrophages and especially the tumor related ones play an essential role on malignant cell proliferation since they are tightly connected with iron absorption and release. The present study was designed to examine the role of iron and desferrioxamine on the immune interaction between human peripheral blood mononuclear cells (PBMC) and human colon carcinoma lines HT-29 and RKO.

Materials and Methods: PBMC co-cultured with either HT-29 or RKO cells were depleted of iron by desferrioxamine (DFO). TNFα, IL-1β, IL-6, IFNγ, IL-2, IL-10 and IL-1ra concentration in the supernatants were examined by ELISA on various combinations of iron depleted and non-depleted PBMC and malignant cells.

Results: DFO added to PBMC incubated with HT-29 cells, caused inhibited secretion of TNFα, IFNγ, IL-1ra and IL-10 whereas IL-1β, IL-6 and IL-2 production was not affected. Addition of DFO to PBMC cultured with RKO cells resulted in reduction of IL-1ra and IL-10 generation only. Depletion of iron from either PBMC or malignant cells and the addition of iron affected differently cytokine production.

Conclusions: Both iron and DFO affect inflammatory cytokine production by human PBMC and intervene in the cross-talk between immune and colon cancer cells from two lines examined. The study enlightens the way iron and DFO may modulate tumorigenesis.

Introduction

The role of iron as a basic element for both normal function of the organism and as a protector from infections has long been established[1]. Studies have shown that there is a close relationship between iron content in macrophages and their ability to interfere with inflammation and infections[2]. Iron homeostasis is controlled by a number of factors, the prominent being hepcidin and ferroportin. While the first one blocks iron transfer through the cell membrane, thus preventing excessive absorption, the second increases iron influx through the membrane[3,4]. Noteable, iron chelators are likewise connected with iron physiology and with inflammation. Visseren et al.[5] have reported that epithelial cells incubated with iron showed an increased production of the pro-inflammatory cytokine IL-6, a process being blocked by co-incubation of the cells with desferrioxamine (DFO). Iron homeostasis is important for development and proliferation of cancer cells. It has been reported that malignant cells require more iron which is supplied by transferrin, explicating the fact that carcinoma cells possess more transferrin receptors[6]. Moreover, altered expression of a group of proteins, particularly hepcidin, affects normal iron metabolism with a consequent cellular iron overload and increased treat for cancer[7-11]. On the other hand, chronic iron depletion by repeated phlebotomy was linked with reduced cancer risk and mortality[11]. Tumor associated macrophages may affect iron homeostasis especially when they infiltrate breast tumors and it is related to increased infiltration of ferritin-loaded macrophages at the tumor environment, while cancer cells showed decreased ferritin expression[12]. According to Jung et al.[13] tumor associated macrophages regulate or alter iron metabolism by several mechanisms, including reprogramming the polarization of M2 macrophages that possess tumor promoting functions to M1 phenotypes with anti-tumor activity[13-15]. Furthermore, DFO exerts an anti-proliferative effect on cancer cells[16]. Since in a previous study we have examined the effect of iron on cytokine production by desferrioxamine treated mononuclears[17], the present work was conceived to detect the effect of iron and DFO on the immune dialogue between human
Peripheral blood mononuclear cells (PBMC) and cells from HT-29 and RKO human colon cancer lines.

Materials and Methods

Cell Preparation
Peripheral blood mononuclear cells (PBMC) were separated from venous blood from blood bank donors using Lymphoprep-1077 (Axis-Shield PoC AS, Oslo, Norway) after obtaining an informed consent. 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% heat inactivated fetal bovine serum (FBS), and was designated as complete medium (CM).

Desferrioxamine Preparation
Desferrioxamine mesylate (DFO) (Desferal, Novartis Pharma, Switzerland) was freshly dissolved in saline at a concentration of 10 mM and further dilutions were prepared in saline. DFO was added to cultures at a final volume of 10µl/ml.

Colon cancer cell lines
HT-29 and RKO human colon cancer cell lines were obtained from American Type Culture Collection, Rockville, MD. The cells were maintained in CM containing MCOY’S 5A medium and modified eagle medium (MEM- Biological Industries Co, Beth-Haemek, Israel) respectively, supplemented with 10% 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.

DFO and cell viability
0.1 ml of PBMC suspension (2 x 10⁶/ml in CM) was added to each of 96 well plates. DFO was used at concentrations of 0, 25 µM, 50 µM and 100 µM. Incubation was carried on for 24 hrs at 37°C in a humidified atmosphere containing 5% CO₂. At the end of the incubation period, the supernatants were removed by centrifugation at 450 g for 10 min, the supernatants were collected and kept at -70°C until assayed for cytokine content.

Effect of DFO on cell proliferation
Since PBMC do not proliferate unless they are exogenously stimulated, the effect of DFO was determined on HT-29 and RKO cell proliferation only, using XTT cell proliferation assay kit (Biological Industries, Beith Haemek, Israel). Briefly: 0.1 ml aliquots of HT-29 or RKO cell suspension (2 x 10³/ml of appropriate CM) were added to each one of 96 well plates and incubated for 24 hrs at 37°C in a humidified atmosphere containing 5% CO₂. At the end of the incubation period 0.1 ml of 10% trypan blue dye was added to each well and the number of viable, as well as dead cells was detected.

Effect of iron on cytokine production by DFO treated HT-29, RKO or PBMC
Another set of experiments was carried out to deplete iron from cancer cells or PBMC. Briefly, 0.5 ml of colon cancer cells (HT-29 or RKO) (4 x 10⁵/ml) suspended in appropriate CM were seeded in each of 24 well plates and were incubated for 150 min at 37°C in a humidified atmosphere containing 5% CO₂ in the absence or presence of DFO at a concentration of 100 µM. At the end of the incubation period, the supernatants were removed and replaced by 0.5 ml of fresh CM and 0.5 ml of PBMC suspension (4 x 10⁶/ml of CM) were added to each well. The cultures were incubated for additional 24 hrs at 37°C in the absence or presence of iron ([Venofer, a hydroxide sucrose solution],Vifor International, St. Gallen, Switzerland) at 100 µg/dl.

Alternatively, PBMC (4 x 10⁶/ml of CM) were incubated without or with 100 µM of DFO for 150 min at 37°C in a humidified atmosphere containing 5% CO₂. At the end of the incubation period the cells were sedimented by centrifugation at 450 g for 10 min and washed twice with saline. Then, the cells were suspended in CM and 0.5 ml aliquots were added to each well of 24 well plates containing 0.5 ml of either HT-29 or RKO cells at 4 x 10⁶/ml. Plates were incubated for additional 24 hrs in the absence or presence of 100 µg/dl iron, as described above.

At the of the incubation periods the cells were removed and the supernatants were collected and kept at -70°C until assayed for cytokine content.

Cytokine content in the supernatants
The concentration of the following cytokines: TNFα, IL-1β, IL-6, IFNγ, IL-2, IL-10, and IL-1ra in the supernatants were tested using ELISA kits specific for these cytokines (Biosource International, Camarillo, CA), as detailed in the guideline 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.

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 DFO on cytokine secretion by PBMC induced by colon cancer cells. SAS vs 9.4 were used for this analysis. Paired t-test was applied to compare between the level of cytokines produced with various concentrations of DFO and that found in control cultures. Probability values of p < 0.05 were considered as significant. The results are expressed as mean ± SEM.

Results
Effect of DFO on cancer cell proliferation
DFO added to HT-29 cells at the concentrations tested had no effect on their proliferation as examined by XTT test (F₃, 35 = 0.16, p = 0.924). However, the proliferation of RKO cells was significantly inhibited (F₃, 35 = 6.52, p = 0.004) and was reduced by 16%, 20% and 17% at DFO concentrations of 25, 50 and 100 µM respectively (p < 0.003, Table 1).

Effect of DFO on cytokine production
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Table 1: Effect of DFO on cell proliferation (XTT test).

<table>
<thead>
<tr>
<th>DFO</th>
<th>HT-29- induced</th>
<th>RKO-induced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absorbance at 450 nm</td>
<td>P*</td>
</tr>
<tr>
<td>0</td>
<td>1841 ± 115</td>
<td>2118 ± 44</td>
</tr>
<tr>
<td>25 µM</td>
<td>1832 ± 100</td>
<td>NS</td>
</tr>
<tr>
<td>50 µM</td>
<td>1868 ± 89</td>
<td>NS</td>
</tr>
<tr>
<td>100 µM</td>
<td>1767 ± 122</td>
<td>NS</td>
</tr>
</tbody>
</table>

HT-29 or RKO cells were incubated without (0) or with DFO at concentrations as indicated. After 24 hrs, cell proliferation was tested using XTT test as described in Materials and Methods section. The results are expressed as Mean ± SEM of 9 experiments. P* value represents statistically significant difference from cells incubated without DFO. NS - not statistically significant.

Effect of DFO and iron on pro-inflammatory cytokine production.

TNFα

DFO caused an inhibition of TNFα production by HT-29 induced PBMC (F3, 18 = 14.56, p < 0.0001), whereas that induced by RKO cells was not affected (F3, 18 = 2.21, p = 0.121). At DFO concentrations of 25, 50 and 100 µM the production of TNFα induced by HT-29 cells was reduced by 22%, 24% and 11% respectively (p < 0.01, Table 2).

Table 2: Effect of DFO on pro-inflammatory cytokine production.

<table>
<thead>
<tr>
<th>DFO µM</th>
<th>TNFα, ng/ml (n = 7)</th>
<th>IL-1β, ng/ml (n = 7)</th>
<th>IL-6, ng/ml (n = 7)</th>
<th>IFNγ, ng/ml (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM P* Mean ± SEM P* Mean ± SEM P* Mean ± SEM P*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.63 ± 0.06 = 8.88 ± 0.94 27.12 ± 0.85 1.76 ± 0.29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>0.49 ± 0.06 &lt; 0.002 8.59 ± 9.35 NS 25.39 ± 0.26 NS 1.38 ± 0.32 = 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.48 ± 0.05 &lt; 0.001 8.75 ± 0.90 NS 25.50 ± 0.29 NS 1.48 ± 0.30 = 0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.56 ± 0.06 &lt; 0.01 8.92 ± 0.98 NS 25.17 ± 0.55 NS 1.49 ± 0.30 &lt; 0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PBMC were incubated for 24 hrs with HT-29 or RKO colon cancer cells in the absence (0) or the presence of DFO at concentrations as indicated. The level of cytokines in the supernatants was tested by ELISA. The results are expressed as Mean ± SEM of 7 experiments. P* value represents statistically significant difference from cells incubated without DFO. NS - not statistically significant.

IL-1β

The secretion of IL-1β by PBMC induced by both colon cancer cells was not affected significantly by 24 hrs of incubation with the above mentioned concentrations of DFO (F3,18 = 0.86, p = 0.48 for HT-29-induced IL-1β secretion and F3,18 = 0.71, p = 0.56 for that induced by RKO cells, (Table 2).
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IL-6

24 hrs of incubation with DFO added at all concentrations had no significant effect on the secretion of IL-6 by PBMC stimulated with either HT-29 cells (F3, 18 = 2.73, p = 0.0744) or by RKO cells (F3, 18 = 1.96, p = 0.15, (Table 2).

IFNγ

IFNγ production by PBMC stimulated with HT-29 cells was inhibited following 24 hrs of incubation with DFO (F3, 18 = 5.24, p = 0.009). At DFO concentrations of 25, 50 and 100 µM it was lowered by 21.5%, 16% and 15%, respectively (p < 0.03). IFNγ secretion by PBMC stimulated with RKO cells was not affected by addition of DFO (F3, 18 = 1.69, p = 0.204) (Table 2). The production of IFNγ by PBMC incubated with HT-29 cells exposed to DFO followed by its removal was lower by 37% (p < 0.001) and remained reduced by 39% (p < 0.002) after iron addition (Table 4). IFNγ secretion by PBMC exposed to DFO followed by its removal and stimulated by HT-29 cells remained slightly lower by 5% (p < 0.05) compared to the control and was further reduced by 25% (p < 0.03) following addition of iron (Table 5). IFNγ secretion by PBMC induced by RKO cells was not affected by any of the procedures carried out in this study (Tables 4 and 5).

Effect of DFO and iron on anti-inflammatory cytokine production

IL-10

A dose dependent inhibition of IL-10 production by HT-29 and RKO stimulated PBMC was found when DFO was added (F3, 18 = 51.43, p < 0.0001 and F3, 18 = 10.6, p < 0.001 and F3, 18 = 3.31, p < 0.05, respectively). IL-10 secretion by PBMC induced by HT-29 cell was lowered by 19%, 14% and 14% (p < 0.03) in the presence of DFO at 25, 50 and 100 µM, and that induced by RKO cells was reduced by 13% (p = 0.01) at 100 µM DFO only (Table 2). The secretion of IL-10 induced by DFO treated HT-29 or RKO cells or by DFO-treated PBMC exposed to malignant cells, did not differ significantly from that of the controls, when DFO was removed from colon cancer cells before exposure to PBMC (Table 4) or from PBMC before stimulation with cancer cells (Table 5). Secretion of IL-1ra was not affected by addition of iron to these cultures (Tables 4 and 5).

Table 3: Effect of DFO on anti-inflammatory cytokine production.

<table>
<thead>
<tr>
<th>DFO µM</th>
<th>IL-10 ng/ml (n=7)</th>
<th>IL-1ra ng/ml (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HT-29- induced</td>
<td>RKO-induced</td>
</tr>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>P*</td>
</tr>
<tr>
<td>0</td>
<td>1.32 ± 0.14</td>
<td>0.82 ± 0.07</td>
</tr>
<tr>
<td>25</td>
<td>0.61 ± 0.07</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>50</td>
<td>0.61 ± 0.06</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>100</td>
<td>0.57 ± 0.07</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

PBMC were incubated for 24 hrs with HT-29 or RKO colon cancer cells in the absence (0) or the presence of DFO at concentrations as indicated. The level of cytokines in the supernatants was tested by ELISA. The results are expressed as Mean ± SEM of 7 experiments. P* value represents statistically significant difference from cells incubated without DFO. NS - not statistically significant.

Table 4: Effect of DFO and iron on cytokine secretion.

<table>
<thead>
<tr>
<th>DFO 100µM</th>
<th>TNFα, ng/ml (n = 6)</th>
<th>IFNγ, ng/ml (n = 6)</th>
<th>IL-10, ng/ml (n = 6)</th>
<th>IL-1ra, ng/ml (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>P*</td>
<td>Mean ± SEM</td>
<td>P*</td>
</tr>
<tr>
<td>HT-29-induced</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.76 ± 0.08</td>
<td>1.11 ± 0.09</td>
<td>1.23 ± 0.13</td>
<td>2.07 ± 0.05</td>
</tr>
<tr>
<td>DFO</td>
<td>0.73 ± 0.06</td>
<td>NS</td>
<td>0.78 ± 0.06</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>DFO+ Iron</td>
<td>0.59 ± 0.07</td>
<td>=0.02</td>
<td>0.68 ± 0.03</td>
<td>&lt; 0.002</td>
</tr>
<tr>
<td>RKO-induced</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.71 ± 0.07</td>
<td>2.04 ± 0.38</td>
<td>0.84 ± 0.11</td>
<td>1.94 ± 0.20</td>
</tr>
<tr>
<td>DFO</td>
<td>0.77 ± 0.07</td>
<td>NS</td>
<td>2.49 ± 0.51</td>
<td>NS</td>
</tr>
<tr>
<td>DFO+ Iron</td>
<td>0.69 ± 0.07</td>
<td>NS</td>
<td>1.72 ± 0.27</td>
<td>NS</td>
</tr>
</tbody>
</table>

HT-29 or RKO colon cancer cells were incubated for 2.5 hrs in the absence (0) or presence of DFO at 100 µM. At the end of the incubation p-
PBMC it has been examined and reported in a previous work[17]. In short, DFO caused an enhanced dose dependent production of IL-1β, IL-6 and IL-1ra, whereas that of IL-2 and IFNγ was lowered. Addition of iron reversed the effect of DFO as for the secretion of IL-2 and IL-10 only.

The next step in our study was to evaluate the cell type that its iron depletion affects the cross talk between PBMC and colon carcinoma cells. For this goal we carried out two experiments. One, to examine cytokine production by iron depleted PBMC incubated with carcinoma cells and the second – cytokine production by PBMC incubated with iron depleted malignant cells. Iron depleted PBMC incubated with HT-29 non-depleted cells revealed inhibited secretion of IFNγ only. The capacity of RKO cells for cytokine production was not affected in this set of experiments. On the other hand, when non-depleted PBMC were incubated with DFO depleted HT-29 cells, there was a decreased generation of TNFα and IL-10 whereas incubation of PBMC incubated with iron depleted TKO cells caused a marked increase of the anti-inflammatory IL-10.

An additional line of experiments was designed to detect the effect of iron added to incubation mixtures containing either depleted PBMC with non-depleted malignant cells or non-depleted PBMC with DFO depleted cancer cells. Here again, the results were cell-dependent. While addition of iron to suspension containing iron depleted PBMC and non-depleted HT-29 cells caused an inhibited secretion of IFNγ and an elevated IL-10 production, the same procedure using RKO cells induced an increased generation of IL-10 only. Iron added to cell suspensions containing non-depleted PBMC with depleted HT-29 cancer cells resulted in a decreased secretion of TNFα, IFNγ and IL-10 by the immune cells, whereas addition of iron to non-depleted PBMC with DFO depleted RKO cells resulted in increased production of IL-10.

The findings in the present study demonstrate that both iron and DFO may affect the immune balance between PBMC and colon carcinoma cells from the two lines used in this work. Studies have shown that DFO may modulate cytokine production by a number of cytokine producing cells[16-18]. Indeed, addition of DFO to PBMC incubated with HT-29 cancer cells caused inhibition in TNFα, IFNγ, IL-1ra and IL-10 secretion, i.e. a suppressed expression of several pro- and anti-inflammatory cytokines examined in the study. However, when the same experiment was carried out with RKO stimulated PBMC this effect was observed only on the generation of IL-1ra and IL-10. These results point out to the capacity of DFO to affect differently the immune cross-talk between PBMC and colon cancer cells from the two lines used in this work. Studies have shown that DFO may modulate cytokine production by a number of cytokine producing cells[17] and it has a marked anti-cancer effect[19]. A pronounced blockade of IL-6 production was one of the noticeable findings in pigs undergoing acute hepatic ischemia followed by re-perfusion[20]. It has been reported that DFO was able to enhance expression of IFNγ by cells of hepato-cellular carcinoma lines with a consequent anti-proliferative effect and induction of apoptosis[21,22]. Similar findings have been observed when colon cancer cells were treated with DFO[23]. Accordingly, it has been suggested that iron chelators may serve as an additional medication against cancer[19,24,25]. In our hands DFO, at all three concentrations applied in the study, exerted a significant proliferation inhibition on RKO cells only. As for the effect of DFO on human
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and colon carcinoma cells from the two lines. The existence of a cross talk between immune and cancer cells expressed by altered cytokine production has been well documented\cite{26-28} and has been shown to be affected by a large number of substances and chemicals\cite{29}.

In short, the results of the study display the role that both iron and desferrioxamine exert on the interaction between peripheral blood mononuclear cells and those from two colon cancer lines expressed by a modulation of inflammatory cytokine production. The casual role of iron on the increased generation of the anti-inflammatory cytokine IL-10 by PBMC incubated with malignant cells is clearly demonstrated by the crossing experiments carried throughout the study. Considering the ways that inflammation may induce and elicit cancer development, the findings may clarify one of the mechanisms by which immune cells may control cancerogenesis.

Acknowledgment: We indebted to Ms. Tzippy Shochat, MSc, Statistical Consultant, Rabin Medical Center, Beilinson Hospital, for her important help in the statistical calculations.

References