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Basic Study

Profiles of proinflammatory cytokine levels (Interferon-gamma and Interleukin-2) in patients post allogeneic hematopoietic stem cells transplantation

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Abstract

BACKGROUND

The procedure of allogeneic hematopoietic stem cells transplantation (alloHSCT) is related to the occurrence of such complications as graft-versus-host disease (GvHD) and infections. The pathogeny of acute GvHD is referred to T-lymphocytes, which distinguish alloantigens on host's Antigen Presenting Cells, activate production of Interferon-gamma (IFN-gamma) and Interleukin-2 (IL-2), acquire the immunological effectory cells and damage tissues and organs.

AIM

The aim of the study was to investigate and distinguish the IFN-gamma and IL-2 serum concentrations profiles in 30 days period after allogeneic hematopoietic stem cells transplantation.

METHODS

In the study there were enrolled 62 patients: 30 (48%) male and 32 (52%) female, aged at median 49.5 (19-68) years, after alloHSCT from sibling (n=12) or from unrelated donor (n=50) performed for acute myeloid leukemia (AML) with myeloablative conditioning

(n=26, 42%) and with non-myeloablative conditioning (n=36, 58%). All patients received standard immunosuppressive therapy with cyclosporin-A and methotrexate plus pre-transplant anti-thymocyte globulin in unrelated setting. Blood samples were collected pre-transplant before start and after (on day -1) the conditioning therapy and on days +2,+4, +6, +10, +20, +30 after alloHSCT. The IL-2 and IFN-gamma serum concentrations were determined with use of ELISA assay.

RESULTS

Before statistical review patients were severed into 4 groups according to the inherence of acute GvHD and clinical manifestation of infection. Group I - patients with neither acute GvHD nor infections, n=15 (24%), group II - patients with infections without acute GvHD, n=17 (27%), group III - patients with acute GvHD without infections, n=9 (15%), and group IV – patients with both acute GvHD and infections, n=21 (34%). IFN-gamma concentrations were higher in group II than in other groups on days +20 (p=0.014) and +30 (p=0.008). The POST-HOC tests indicated lower concentrations of IFN-gamma on day +30 in group I (p=0.039) and in group IV (p=0.017) as compared to group II. The level of IL-2 was non detectable in almost all checkpoints.

CONCLUSION

Serum levels of IFN-gamma following alloHSCT progressively escalate. High serum level of IFN-gamma is referred to infectious complications rather than to acute GvHD. Serum concentrations of IL-2 in the generality of patients were undetectable.

INTRODUCTION

The procedure of allogeneic hematopoietic stem cells transplantation (alloHSCT) is exposed to risk of complications such as graft-versus-host disease (GvHD) and infection. The graft-versus-host disease is crucial and potentially fatal complication of allogeneic hematopoietic stem cells transplantation. It is observed both in the case of transplantation from a related and unrelated donor. It occurs in two forms: acute graft-

versus-host disease (aGvHD) and chronic graft-versus-host disease (cGvHD). GvHD was considered to be induced by donor T lymphocytes, which are incited by classic human leukocyte antigens (HLA) in the event of transplantation from a not fully matched donors, or by weak human leukocyte antigens in the case of transplantations from fully matched donors [1,2,3]. The pathophysiological concept of acute graft-versus-host disease created by Ferrara et al. [4] assumes that the pathogenesis of aGvHD involves three consecutive stages: in the first stage, pre-transplantation conditioning treatment involve lesion and stimulation of host tissues with elicitation of proinflammatory cytokines TNF-α and IL-1, and then induction of antigen presenting cells (APC). In the second stage, T lymphocytes, which recognise alloantigens on the host cells, initiate a so-called "cytokine storm", complete with secretion of interferon (IFN) gamma and interleukin (IL) 2, and acquire effector cells of the immune system, and then, in the third stage, the tissues and organs are damaged by the inflammatory process activated by cytokines secrete by their cytotoxic T cells, NK cells and macrophages. IFN-gamma and IL-2 are core cytokines inducing the graft-versus-host reaction by enhanced activation of the immune cells in reply to alloantigens [5,6,7].

MATERIAL AND METHODS

The study included 62 subjects with a diagnosed acute myeloid leukaemia who underwent allogeneic hematopoietic stem cells transplantation at the Department of Hematology and Bone Marrow Transplantation of the Independent Public Clinical Hospital, Medical University of Silesia in Katowice, Poland between 2012 and 2014. The subjects included 30 (48%) males and 32 (52%) females aged between 19 and 68 years (median age 49.5). Time from diagnosis to transplantation was between 4 months and 10 years (median 11 months), while at the moment of transplantation 54 patients (87%) were in the phase of complete remission (CR), 3 patients (5%) in partial remission (PR), and the other did not reach remission. The conditioning treatment was based on the following regimens: TreoFluATG (n=26, 42%), BuCyATG (n=14, 23%), BuCy (n=6, 10%), TreoFlu (n=5, 8%), TBICyATG (n=5, 8%), BuFluATG (n=3, 5%), and in isolated cases

TreoFluThymo, BuFlu, BuCyThymo, where the myeloablative conditioning (MAC) amounted to 42% (n=26) and reduced intensity conditioning (RIC) 58% (n=36). 50 patients (81%) received unrelated donor hematopoietic stem cells transplantation (URDHSCT), and the other patients (n=12, 19%) received sibling hematopoietic stem cells transplantation (sibHSCT). All the patients underwent standard immunosuppressive therapy, including 95% (n=59) cases based on cyclosporin and methotrexate, with addition of antilymphocyte globulin in the case of unrelated donor transplantation. aGvHD was diagnosed based on clinical criteria and was graded according to the Glucksberg scale. Infection complications were diagnosed based on clinical symptoms and findings of the bacteriological tests of the collected materials. Death during hospitalisation within +30 days after alloHSCT was reported in 4 patients (6%). Peripheral blood samples were collected from each patient in the amount of 5 mL per clot at the following time points: before starting conditioning treatment, after its completion (day -1), and after transplantation on days: +2 +4, +6, +10, +20, +30, unless death occurred earlier. The collected blood was immediately centrifuged, and the obtained serum was stored frozen at a temperature of -80 degrees Celsius until the time of analysis. The levels of IFN-gamma and IL-2 in the test material were determined using the ELISA method.

Statistical analysis

The statistical analysis of the study was executed by a biomedical statistician. The study population was characterised by presentation of the percentage distribution of the qualitative variable variants, while in the case of quantitative variables, the median value and range were used. The levels of the analysed cytokines were initially analysed at all checkpoints, by estimating the mean, median, standard deviation (SD) and standard error of the mean (SEM), and by defining the minimum and maximum value. Due to significant right-skewed cytokine distribution, resulting in rejecting the hypothesis of their normal distribution verified by the Shapiro-Wilk test, further analysis used the median as the measure of central tendency, and the interquartile

range was used as the measure of dispersion. Moreover, non-parametric procedures were used to test statistical hypotheses. Due to multiple 0 values of the analysed cytokines, the variables defining their levels at the test time points were categorised not only against the cut-off levels defined by the set manufacturer (for IL-2 7pg/mL, for IFN-gamma 5pg/mL), but also against the value 0. The entire statistical analysis was performed at the significance level of 0.05. The results for which p was lower than 0.05 ($p<0.05$), with 95% confidence interval(CI) were considered statistically significant.

RESULTS

Hematological recovery after stem cell transplantation was observed in 61 (98%) patients, and it was as follows: regarding WBC (>1.0 G/L) - median time to recovery day +15 (range 11-25), ANC (>0.5 G/L) – day +17 (11-27).

aGvHD manifestation after alloHSCT was observed in 30 (48%) patients - median time to manifestation day +17 (range 8-29). aGvHD was diagnosed with the following grades: I - among 26 (42%) patients, II - among 3 (5%), III – among 1 (2%), IV – not recorded, and included the following organs: skin – 28 (45%) patients, bowel– 3 (5%), liver – not recorded. The remaining 32 patients (52%) did not show aGvHD symptoms. An infectious complication independently of its etiology appeared in 38 (61%) patients– median time to the appearance of the first incident- day +9 (range 0-27). Mucositis appeared in 26 (42%) patients – median time to the occurrence +1 day (range 0-20) (Table 1).

21 patients (70%) with aGvHD had also bacterial and/or fungal and/or viral infection - the median time to the first infection incident was day +10 (range 1-27). Mucositis was reported in 15 patients (50%) – median time to the occurrence day +2 (0-8), which, however, was not recorded as significantly different in comparison with the group of patients without aGvHD (Table 1).

Assessment included kinetics of IFN-gamma changes after alloHSCT in the groups with and without aGvHD at consecutive time points, including assessment before and after conditioning treatment (Figure 1).

No differences regarding the IFN-levels tested before and after conditioning treatment were observed between the subjects with and without aGvHD. In the group of patients without aGvHD manifestations, a significant increase in the cytokine level was observed on day +20. The achieved level was maintained in the next measurement. In patients with aGvHD, an increase in the IFN-gamma level in relation to the level before the conditioning treatment was observed on day +6. At this measurement point, the higher IFN-gamma level in the aGvHD group in relation to the group without aGvHD was the most pronounced. This advantage could also be observed on day +10. At the next measurement point, a shift was observed regarding the IFN-gamma level, which was now higher in patients without aGvHD. The above observation may be correlated with the disease onset, whose median was on day +17. However, the difference in the IFN-gamma level before and during manifestation of aGvHD symptoms, although indicated a decrease in the cytokine level, was not statistically significant.

The assessment also included the kinetics of IFN-gamma changes after alloHSCT in the groups of patients with and without infections at consecutive time points, including assessment before and after the conditioning treatment (Figure 2).

No significant difference in the IFN-gamma level tested before the conditioning treatment was observed between the groups with and without infection, although a tendency to a higher cytokine level was observed in the group without infection. The analysis of the effect of the conditioning treatment on the cytokine level showed its decrease only in the patients whose post-transplantation period was not complicated by infection, which in turn led to the reversal of proportions in both groups. A tendency to a higher IFN-gamma level in the group of patients with infection, observed after the use of conditioning treatment, was maintained during the entire post-transplantation

period, and on day +6 the difference in the IFN-gamma levels reached significance. In the group of patients with infection, an almost continuous increase in the IFN-gamma level was observed during the entire examined period, while on day +20 the concentration was significantly higher than baseline, and was also slightly increased at the next measurement point. In the other patients, the lowest IFN-gamma level revealed after the conditioning treatment showed a growing tendency at subsequent measurements, but this tendency was not as significant as in the group of patients with infection.

Moreover, the median value and the maximum value of the IFN-gamma level was measured for each patient in the post-transplantation period (between +2 and +30). These values were significantly higher in the group of subjects with infection compared with those without infection (median 0.058 vs 0 pg/mL, p=0.043; 19.295 vs 2.260 pg/mL, p=0.002, respectively) (Table 2, Table 3).

The presence of IL-2, tested before the conditioning treatment, was reported only in one patient, who did not develop aGvHD and have no infection as a result of transplantation. The observed value of the IL-2 level was decreased after the use of conditioning treatment to a value below the cut-off level, but was maintained above the value 0, with no increase in the cytokine level in the other patients.

The IL-2 level, tested after alloHSCT, was above the value 0 at least at one measurement point only in 5 patients, including 3 above the cut-off point. None of these patients revealed manifestation of aGvHD symptoms, but 3 out of 5 these patients had infections independently of their etiology, and 2 of them had mucositis. In all the patients, on the day of infection onset, the IL-2 level was undetectable. Figure 3 shows the kinetics of the IL-2 changes in serum of the patients whose level was above 0 at any time during the post-transplantation period.

In the final analysis, the patients were categorised into 4 groups based on the occurrence of aGvHD and infection. Group I: patients (n=15, 24%) revealed no signs of acute GvHD or infection; group II: patients (n=17, 27%) showed complications in the form of infection after alloHSCT, without acute GvHD; group III patients (n=9, 15%)

showed acute GvHD with no infection; and group IV patients (n=21, 34%) showed both acute GvHD, and infection. The analysis of IFN-gamma levels measured at subsequent measurement points showed the occurrence of differences between the groups on days +20 and +30 after alloHSCT (Table 4).

Significantly higher levels of this cytokine were observed in group II on checkpoint days +20 (p=0.014) and +30 (p=0.008) in comparison with the other groups of patients. POST-HOC tests revealed significantly lower IFN-gamma levels on checkpoint day +30 in group I (p=0.039) and IV (p=0.017) in comparison with group II. The IL-2 level was non detectable in majority of patients at all the measurement checkpoints. Figure 4 shows mean IFN-gamma levels before and after alloHSCT in the above-mentioned 4 groups of patients.

DISCUSSION

This paper presents the profiles of IL-2 and IFN-gamma levels depending on the occurrence of acute graft-versus-host disease and infection complications post alloHSCT. In this report, the cytokine level was tested in the early period after alloHSCT covering the first 30 days after transplantation. Before, during and after blood sampling for cytokine determination, the cytokine-producing cells were not stimulated with mitogen substances (LPS (Lipopolysaccharide), PHA Phytohemagglutinin). Therefore the achieved cytokine levels and their profile may be considered as reflecting real values in patients who underwent alloHSCT. The low levels of IFN-gamma and IL-2 revealed in the test before and after alloHSCT may be caused by deep pancytopenia occurring after the conditioning treatment and after the use of ATG (Antithymocyte Globulin), which results in a deficiency of cytokine-producing cells. Moreover, the immunosuppression caused impairment of the hematopoetic cell function and their production of cytokines. Low IFN-gamma and IL-2 levels may to a certain extent indicate successful immunosuppressive treatment. However, beginning with day 20 after alloHSCT, the cytokine levels were gradually increasing, together with progressive reconstitution of the hematopoetic cells.

In the present study, the levels of the analysed cytokines were low and in a small number of patients they exceeded the value of the cut-off point considered as a positive result. This may be related to lymphocyte dysfunction caused by intensive AML treatment. In AML patients, T lymphocytes show genetic and phenotype disorders, as well as impaired function and reduced cell count [8,9]. It was proved that this could be related to a disrupted function of T lymphocyte receptor (TCR) (especially subunit ζ), whose damage causes reduction in the immune defence in leukemias. In addition, T lymphocytes, especially with TCR Vβ, are not fully recovered after induction treatment in AML patients [10,11]. Moreover, long-term antigen stimulation of T lymphocytes leads to "exhaustion" of these cells, which means that T lymphocytes lose their ability to secrete such cytokines as: IL-2, TNF-α and IFN-gamma, proliferate and induce cytotoxic reactions [12,13,14].

In their publication, Sadeghi et al. [15] presented a murine model of aGvHD based on conditioning with the use of high-dose chemotherapy. The animals underwent conditioning, and then were divided into two groups. Group I comprised animals that underwent allogeneic transplantation, while group II comprised animals with syngeneic transplantation. The authors did not use immunosuppressive treatment in the post-transplantation period. The period of bone marrow cell regeneration was varied depending on the type of transplantation: in mice after syngeneic transplantation it was more rapid with a shorter duration (onset of recovery on day +1, peak on day +5 and end on day +21, while in mice after allogeneic transplantation there was no complete recovery by day +21). Analysing the collected blood samples, the authors examined reconstitution of the immune cells of mice and the kinetics of the cytokines: IFN-gamma, IL-2 and TNF-α in the early period after transplantation. It was proved that in the mice after allogeneic transplantation, proliferation and maturation of dendritic cells and T CD8+ lymphocytes of the donor was more rapid in comparison with the mice after syngeneic transplantation: day +3 and from day +5 after transplantation, respectively. Analysing the cytokine kinetics, the authors noted a regular increase in the cytokine levels (concerning all cytokines), which corresponded

with the rate of bone marrow cell recovery and the presence of dendritic cells and T lymphocytes in the circulation. The increase in the cytokine level was higher in the mice after allogeneic transplantation with observed aGvHD. It was also observed that the levels of the above-mentioned cytokines, while gradually increasing from a low level on the day of transplantation, reached their peak on day +5 in mice subjected to allogeneic stem cell transplantation which developed aGvHD, followed by a decrease on subsequent days. No such phenomenon was observed in the animals after syngeneic transplantation.

In contrast to the present article, the authors of the above-mentioned study based their study design on a murine model and did not include immunosuppressive treatment. Therefore, the presented kinetics of the cytokine level changes and reconstitution of bone marrow cells were deprived of the blocking effect of immunosuppressive agents on the immune cells and on cytokine secretion. This is reflected by rapid recovery of bone marrow cells that they describe, with a simultaneous increase in the cytokine level. The presented study did not account for the period before the conditioning treatment, and the follow-up period was shorter and amounted to 21 days after transplantation.

A study conducted by Ju et al.[16] analysed cytokine expression at a molecular level and protein expression in 30 patients after allogeneic peripheral blood stem cell transplantation (alloPBSCT). The group of patients enrolled in the study was not homogeneous with regard to the disease being an indication for transplantation. A myeloablative regimen Cy+VP16+TBI was used as conditioning treatment, while ciclosporin A + methotrexate was used as aGvHD prevention. Blood samples were collected from the patients before alloPBSCT, during the occurrence of first aGvHD symptoms and after pharmacological control of the diseases symptoms. Then, the samples were incubated and a solution of lipopolysaccharide and PHA was added, and the level of cytokines: IFN-gamma, IL-2, IL-4, IL-10, IL-12 and IL-18 was measured using the ELISA method. Out of 30 examined patients, 16 patients did not develop aGvHD, 7 patients had grade I aGvHD symptoms, and 7 patients had grade II-IV

aGvHD. Expression of the examined cytokines, especially IL-2 and IFN-gamma, both at the mRNA and protein level, was significantly higher in patients with aGvHD. Its expansion was observed with increased severity of aGvHD, and a significant decrease when the aGvHD symptoms were controlled. In the above study, a correlation between the protein level of the above-mentioned cytokines and aGvHD symptoms was stronger than between their mRNA expression and symptoms of this disease.

The authors of the above report, unlike the authors of this paper, measured the cytokine lever after stimulation with lipopolysaccharides and PHA, which increase cytokine secretion, achieving in this way their high concentration. Moreover, despite standard immunosuppressive treatment, high IL-2 levels (>100 pg/mL) were observed both in the group of patients with aGvHD occurrence and in the group of patients without aGvHD symptoms. In the present study, the IL-2 was below detection limit in almost all patients, regardless of the presence of aGvHD symptoms, and the IFN-gamma level was higher in the group without aGvHD symptoms. Achieving high IL-2 levels in both groups of patients, with or without aGvHD, despite using standard immunosuppression, indicates a possible decisive effect of LPS and PHA stimulation of the immune cells on achieving high cytokine levels. For this reason, the real picture of the kinetics of IL-2 and IFN-gamma level changes in the post-transplantation period in patients developing aGvHD is blurred in the study referred to.

A study conducted by Visentainer et al.[17] included 13 patients after alloHSCT from a fully matched donor, who had their serum cytokine level determined within 15 weeks after alloHSCT using the ELISA method. Only the level of IL-10 and soluble receptor for IL-2 was significantly higher in the group of patients with aGvHD symptoms in comparison with patients without symptoms of this disease. Moreover, the level of soluble receptor for IL-2 increased in a direct correlation with implantation of bone marrow cells and onset of aGvHD symptoms.

As far as the correlation between IFN-gamma and infection complications in patients after alloHSCT is concerned, few available publications present contradictory opinions on this issue. In a study by Gayoso et al.,[18] a group of 26 patients was

examined for a correlation between the IFN-gamma level and the occurrence of CMV infection in the period over 6 months after alloHSCT. The prophylaxis of aGvHD included the use of cyclosporin and methotrexate in patients after myeloablative conditioning, and the use of cyclosporin with mycophenolate mofetil after RIC. If CMV reactivation was observed, the patient was treated with valganciclovir. Blood samples were collected from patients after 6 months of alloHSCT. They were centrifuged and the IFN-gamma level was determined in the supernatant using the ELISA method. The authors proved that in patients with cytomegalovirus reactivation, the IFN-gamma level was higher (>0.2 IU/mL) than in the group where no reactivation was observed.

In another report by Peng et al.,[19] a correlation between proinflammatory cytokines and the occurrence of invasive fungal infection was studied in patients after alloHSCT. The analysis included 47 patients who underwent allo-HSCT due to various hematological diseases, and a control group comprising 40 healthy volunteers. All the subjects received myeloablative conditioning treatment, including 17 patients with ATG, and 30 patients without ATG. The immunosuppressive treatment preventing aGvHD included: tacrolimus with methotrexate and mycophenolate mofetil in 30 patients, and cyclosporin with methotrexate and mycophenolate mofetil in 17 patients. The levels of IL-6, IL-10, IFN-gamma and TGF-β cytokines were determined using ELISA at 1, 2 and 3 months after allo-HSCT. A comparison of the results achieved in patients after alloHSCT and the control group showed that the IL-6 level was gradually increasing and reached the peak value at 2 months after alloHSCT, and then got reduced. At 3 months, however, it was significantly higher in patients after alloHSCT. Similarly, the IL-10 level was increasing in the post-transplantation period, while the TGF-β level was gradually reduced. With regard to the IFN-gamma level, no significant differences were observed between the analysed groups. Moreover, there was no proof for the correlation between invasive fungal infection and changes in the concentration of IFN-gamma and other cytokines.

In the above-mentioned publications, the authors focused their attention on showing a correlation between one selected infection complication and the

concentration of specific proinflammatory cytokines. The study groups were not homogeneous with regard to the disease entity being an indication for alloHSCT. The cytokine levels were determined in the late post-transplantation period with no regard to the effect of GvHD and other complications in the form of infections, mainly bacterial, on the achieved results. In contrast to the present paper the above-mentioned studies reflect the kinetics of cytokine level changes in a later period after alloHSCT in relation to selected infection complications, without accounting for the early post-transplantation period. The present study proves a potential correlation between the IFN-gamma level and the occurrence of infection complications, while the above-mentioned reports present non-consistent data on this issue.

CONCLUSIONS

Higher IFN-gamma levels are more related with infection complications than with aGvHD occurrences. The IFN-gamma level in the group of patients only with acute GvHD, in the group with acute GvHD and infection, and in the group without GvHD and infection is significantly lower than in the group of patients only with infection. Serum IL-2 levels in patients with AML within the first 30 days after alloHSCT are very low and diagnostically insignificant.

ARTICLE HIGHLIGHTS

Research background

The procedure of allogeneic hematopoietic stem cells transplantation (alloHSCT) is related to a risk of such complication as graft-versus-host disease (GvHD) and infections. The graft-versus-host disease is one of the most important and potentially fatal complications of allogeneic hematopoietic stem cells transplantation. The patogenesis is associated with „the cytokine storm".

Research motivation

There is small amount of medical data about the cytokine profiles in patients after allogeneic stem cells transplantation. In this report, the cytokine level was tested in the early period after alloHSCT covering the first 30 days after transplantation.

Research objectives

Before, during and after blood sampling for cytokine determination, the cytokine-producing cells were not stimulated with mitogen substances. Therefore the achieved cytokine levels and their profile may be considered as reflecting real values in patients who underwent alloHSCT.

Research methods

In the study there were enrolled 62 patients after alloHSCT performed for acute myeloid leukemia (AML). All patients received standard immunosuppressive therapy with cyclosporin-A and methotrexate plus pre-transplant anti-thymocyte globulin in unrelated setting. Blood serum samples were collected pre-transplant before start and after (on day -1) the conditioning therapy and on days +2,+4, +6, +10, +20, +30 after alloHSCT. Samples were not stimulated with mitogen substances. The IL-2 and IFN-gamma serum concentrations were determined with use of ELISA assay.

Research results

In the final analysis, the patients were categorised into 4 groups based on the occurrence of aGvHD and infection. Significantly higher levels of this cytokine were observed in group II on days +20 (P = 0.014) and +30 (P = 0.008) in comparison with the other groups of patients.

Research conclusions

Higher IFN-gamma levels are more related with infection complications than with aGvHD occurrences. Serum IL-2 Levels in patients with AML within the first 30 days after alloHSCT are very low and diagnostically insignificant.

Research perspectives

There is a need to conduct further studies to create more spacious cytokine profile.

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The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the Medical University of Silesia in Katowice which issued its consent no. KNW/0022/KB1/71/I/12 on 03/07/2012.

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