The Effect of Interleukin-2 on Natural Killer Activity of Bone Marrow and Peripheral Stem Cell Products from non-Hodgkin's Lymphoma Patients

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The activity of natural killer (NK) cells of peripheral stem cell (PSC) products from 24 patients with non-Hodgkin's lymphoma (NHL) were examined both before and following 5 days co-incubation with IL-2. This study showed that the PSC products prior to IL-2 incubation had higher NK activity than bone marrow (BM) products ($p<0.001$), but with activity similar to normal peripheral blood leukocytes (PBL). Following IL-2 activation, PSC and BM cells had lower NK activity compared to PBL from normal donors ($p<0.001$). When leukocytes were cultured in the presence of IL-2 for 5 days, the NK activity of BM cells was significantly lower than both PBL and PSC ($p<0.01$), but there was no difference between PBL and PSC. The NK activities of PSC, BM and PBL were increased 176, 14 and 40 folds compared to cells prior to IL-2 culture (all of these $p<0.001$). As these results show, ex vivo *threat of PSC before PSC transplantation is capable of to increase defective NK-cytotoxicity. [Turgut Özal Tıp Merkezi Dergisi 1996;3(3):177-182]*

***Key Words**: Non-Hodgkin lymphoma, IL-2, NK cytotoxicity, bone marrow cell, peripheral stem cell*

Non-Hodgkin lenfomalı hastalardan elde edilen periferik stem cell ve kemik iliği hücrelerinin natural killer aktivitesi üzerine interleukin-2'nin etkisi

Non-Hodgkin lenfomalı 24 hastadan elde edilen periferik stem cell (PSC) ürünlerinin natural killer (NK) hücre aktivitesi, IL-2 ile kültürden önce ve 5 gün IL-2 ile birlikte kültür yapılarak incelendi. Bu çalışmaya göre PSC ürünleri IL-2 ile stimüle edilmeden önce kemik iliği (BM) ürünlerinden daha yüksek NK cell aktivitesine sahip ($p<0.001$) iken normal periferik kan lökositleri (PBL)'ne eşdeğer aktivite gösterdi. İnterleukin-2 aktivasyonundan sonra, PSC ve BM hücrelerinin NK hücre aktivitesi, normal donörlerden elde edilen PBL'ye göre daha düşüktü ($p<0.001$). Lökositler IL-2 ile 5 gün kültür yapılınca, BM hücrelerinin NK aktivitesi hem PBL hem de PSC'den anlamlı olarak daha düşük ($p\leq0.01$) iken PBL ve PSC arasında farklılık gözlenmedi. IL-2 ile kültür öncesine göre PSC, BM ve PBL'nin NK aktivitesi, kültürden sonra, sırasıyla 176, 14 ve 40 kat artma gösterdi (tümü için $p<0.001$). Bu sonuçlar, PSC transplantasyonundan önce PSC'in in vitro IL-2 ile muamele edilmesi yoluyla defektif NK-sitotoksisitesini düzeltilebileceğini göstermektedir. [Journal of Turgut Özal Medical Center 1996;3(3):177-182]

***Anahtar Kelimeler**: Non-Hodgkin lenfoma, IL-2, NK sitotoksisitesi, kemik iliği hücreleri, periferik stem cell*

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Recent studies have shown that the immune function of PBL following autologous bone marrow (ABM) transplantation and peripheral stem cell (PSC) transplantation is significantly suppressed compared to normal PBL (1-6). Following stem cell transplantation, patients undergo a prolonged period of T cell immunodeficiency including their immune responses to antigen and mitogen induced T cell proliferation and IL-2 production (7). In contrast to these observations, lymphocyte proliferative response to high concentration of IL-2 in the absence of antigen stimulation has been reported to normalize more rapidly following transplantation and to be associated with the presence of normal or increased numbers of circulating NK (cells) (8).

IL-2 supports the proliferation of T cells, stimulates the production of cytokines, supports the proliferation of NK cells, augments lymphokine activated killer (LAK) cell activity, enhances monocyte cytotoxicity, and stimulates the proliferation of B cells (9,10). However, IL-2 supported proliferation is dependent on the presence of one of several membrane receptors on T, NK and B lymphocytes. During steady state condition, 10% of NK cells as compared to 1% of the total peripheral blood mononuclear cell (PBMC) cellular population express IL-2 receptors (11,12). Furthermore, IL-2 receptors are also found on monocytes (12,13). However, within steady state peripheral blood only NK cells express the high affinity IL-2 receptors necessary for proliferation as compared to other cell types including T cells which must have their receptors upregulated by antigen stimulation (9,14,15).

The aim of this study was to evaluate the response of NK cells to IL-2 in PSC in BM product from NHL patients. Because IL-2 have a pivotal role in lymphocyte proliferation, we have examined the ability of IL-2 to extend the activity of NK-cells from both BM and PSC products compared to that of the PBL from healthy donors using short-term (5 day) cultures with IL-2.

MATERIALS AND METHODS

Patients. Between August 1995 and April 1996, a total of 24 consecutive intermediate grade NHL patients who were candidates for high dose therapy (HDT) and PSC transplantation (n=14) or autologue BM transplantation (n=10) at the University of Nebraska Medical Center (UNMC) were entered into these studies. Written informed consent for stem cell collection and autologous transplantation was obtained from each patient. Peripheral blood progenitor cells were mobilized with granulocyte-monocyte colony stimulating factor (GM-CSF) by intravenous administration of 250 $\mu g/M^2$ and a target dose of $6.5x10^8$ mononuclear cells/kg body weight, and samples were collected and cryopreserved. A minimum of three apheresis sessions were performed beginning at three or more days after the initiation of GM-CSF administration. Following stem cell transplantation, all patients received GM-CSF until their absolute neutrophil count was $\geq 500/mm^3$ on two consecutive days. BM products were collected according to protocol established at the University of Nebraska Medical Center. All samples were obtained using protocols approved by the Institutional Review Board of UNMC. In addition, PBL were obtained from 20 normal healthy volunteer donors.

Cell isolation. The PSC, BM or PB was diluted 1:1 (1:2 for PSC products) in Hanks Balanced Salt Solution (HBSS) (Gibco BRL, Grand Island, NY), layered on Ficoll Hypaque (Organon Teknika, Durham, NC) and centrifuged for 20 minutes at 1400 rpm. After centrifugation, the aqueous layer was removed and the mononuclear cell layer transferred to another tube. The cells were then washed twice in HBSS and adjusted to $4x10^6$/ml in RPMI-1640 (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Hyclone, Logan UT), 10 mM HEPES (Research Organics, Cleveland, OH), 40 μg/ml Gentamycin (Gibco BRL) and 2 mM L-Glutamine (Gibco BRL).

Cell culture. K562, a NK-sensitive, human chronic myeloid leukemia cell line in blast crisis (ATCC #CCL243) was grown in RPMI-1640 medium supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS), 2 mM L-glutamine and 40 μg/ml Gentamycin.

Five days IL-2 co-culture. Fresh PSC's, BM cells, and PBLs were cultured in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine and 40 μg/ml gentamycin. For each experiment, a T-25 tissue culture flasks (Costar, Cambridge, MA) was established with $1x10^6$ cells per ml. These flasks were supplemented with recombinant human IL-2 (Chiron Corporation,

Emeryville, CA) (specific activity $3x10^6$ units/mg) at a final concentration of 100 IU/ml. Cultures were incubated at 37°C in a 5% CO_2 incubator for 5 days, after which period cells were harvested and tested for cytotoxicity.

NK cytotoxicity assay. Cytotoxic activity was assayed against ^{51}Cr-labeled K562 tumor target cells before and after in vitro activation with IL-2. The target tumor cells were labeled with 50 µCi of Na_2

$$\% \text{ Spesific Lysis} = \frac{\text{Experimental Release (ER) - Spontaneous Relea}}{\text{Total Release (TR) - Spontaneous Release (S}}$$

$^{51}CrO_4$ (Amersham Corp., Arlington Heights, IL). K562 cells (~10^7 cells) were incubated with 50 µCi ^{51}Cr in 1 ml RPMI-10 for 1 hour at 37°C. After incubation, cells were washed and reincubated in 5 ml RPMI-10 for 45 minutes. Then, ^{51}Cr-labeled target cells were rewashed 2 times, cell viability was assessed by trypan blue exclusion and adjusted to $1x10^5$ cells/ml. In the studies of fresh (prior to IL-2 culture) effector cells in 100 µl of RPMI-10 were added to the wells of 96-well plates to achieve effector-target (E:T) cell ratios of 40:1, 20:1, 10:1 and 5:1 for NK assay. After culture with IL-2, E:T ratios were adjusted to 10:1, 5:1, 2.5:1 and 1.25:1. Effector and target cells (100,000/well) were mixed in round-bottom 96-well microtiter plates in triplicate and incubated for 4 hours at 37°C. Spontaneous and total release controls were prepared for each assay with culture medium alone or 2% Triton X. At the end of incubation, the supernatants were harvested using a Skatron supernatant harvester system (Skatron A.S. Libergysen, Lier, Norway). The ^{51}Cr released from lysed cells was measured as count per minute (cpm) using a Packard, Cobra II gamma counter (Packard Instrument Company, One State Street, Meriden, CT). The percent target cells lysed (activity of LAK/NK cells) was calculated with the following formula:

ER was defined as the mean cpm of released ^{51}Cr in supernatant from effector plus target cells. TR was defined as the mean cpm of total ^{51}Cr incorporated by the combination to target cells plus 2% Triton-X, and SR was defined as the mean cpm of ^{51}Cr detected in the supernatant of target cells clone (includes target cells plus RPMI-10). The SR was < 5% of maximum incorporated ^{51}Cr.

To normalize the results from the cytotoxicity assay using different E:T cells ratios, the percent cytotoxicity was converted to lytic units (LU). One LU was defined as the number of effector cells required to cause 20% specific ^{51}Cr release from $1x10^5$ target cells; the cytotoxicity in LU/10^7 effector cells was computed (16-18).

Statistics. Results of experimental data obtained from multiple experiments were reported as mean ± standard error of the mean (SEM). Significance levels were determined by the Student's unpaired t-test analysis using SPSS for Windows®.

RESULTS

Patients. The median age of the PSCT patients was 45 years (range 32 to 68 years) and 49 years (range 34 to 68 years) for the autologous BMT patients. The PSCT patients were 8 males while the BMT patients were 7 males.

NK cell activity. The comparison of NK activity within the cells of BM and GM-CSF-mobilized PSC products from NHL patients revealed significant differences. BM cells had significantly lower NK cell activity (Figure 1) compared to PSC products and normal PBL at a 40:1 E:T ratio ($p<0.001$ and $p< 0.001$, respectively). In contrast, NK cell activity from the cells within the PSC products was not significantly different from that observed within normal PB leukocytes ($p=0.269$). A comparison of lytic units (20% cytotoxicity per 10^7 cells) showed that normal PBL had 1.5 lytic units (20% cytotoxicity per 10^7 effector cells), whereas the cells from PSC products had 0.5 lytic units and the BM cells had -0.4 lytic units. Figure 1b shows that NK cell activity increased following co-culture of all of the cell products in IL-2 at 100 IU/ml for five days. However, similar to the fresh products, a significantly lower level of NK cell activity was found in the BM as compared to the PSC products ($p<0.01$) and normal PBL ($p<0.01$). Following co-culture of the BM cells, six lytic units was observed which is in contrast to the 88 lytic units observed following co-culture of PSC cells and the 60 lytic units observed following co-culture of normal PBL. No significant difference at either an E:T ratio of 10:1 or 5:1 was observed in the NK cell activity of GM-CSF mobilized PSC stem cell products and normal PBL.

DISCUSSION

PSCT has been used increasingly as an alternative to ABMT after myeloablative therapy in treatment of malignancies. Perhaps the greatest area of apparent improved efficacy with PSCT as compared to ABMT is the more rapid myeloid (19) and immunologic reconstitution (20) following PSCT as compared to BMT (20,21). The former advantage is somewhat controversial, as the patients receiving ABMT have not received mobilized BM and this is not felt to be a valid comparison. The apparent more rapid immunologic reconstitution following PSCT has been an interesting observation (20). It appears that both ABMT and PSCT may results in the reinfusion of tumor cells from the harvested stem cell products. Cervantes *et al.* (22) have been analyzed the NHL patients which they treated with ABMT over an 11-year period, and reported in a series of 34 patients that tumor involvement of BM was 68%. Brada *et al.* (23) reported that 30% of patients with histologically normal BM had circulating lymphoma cells. PSC harvested from patients during the recovery phase from chemotherapy-induced neutropenia are an alternative source of pluripotent hematopoietic progenitor cells with the possible added advantage of a lower tumor cell contamination (24). Recently, there has been several studies using purged stem cell products which have suggested that the removal of tumor cells from within the products results in a significant prolongation of failure free and overall survival (25). However, regardless of the use of purged products many of the patients still relapse due to the inability of the conditioning regimen to fully remove all the tumor cells. Therefore, additional adjuvant therapeutic strategies are needed to improve the therapeutic efficacy of these different approaches. One such approach that is currently focused upon is the use of IL-2 augmented stem cell products (20) or the use of IL-2 following transplantation (21). Therefore, we undertook a study of NK cell augmentation by the culture of PSC products with IL-2 and examined the T cell and cytokine responses compared this with the response of BM cells and normal PB leukocytes. The latter is particularly important as recent studies from our laboratory have suggested that there are suppressor cells within PSC products which may significantly impact on the proliferative and T cell response of PSC products.

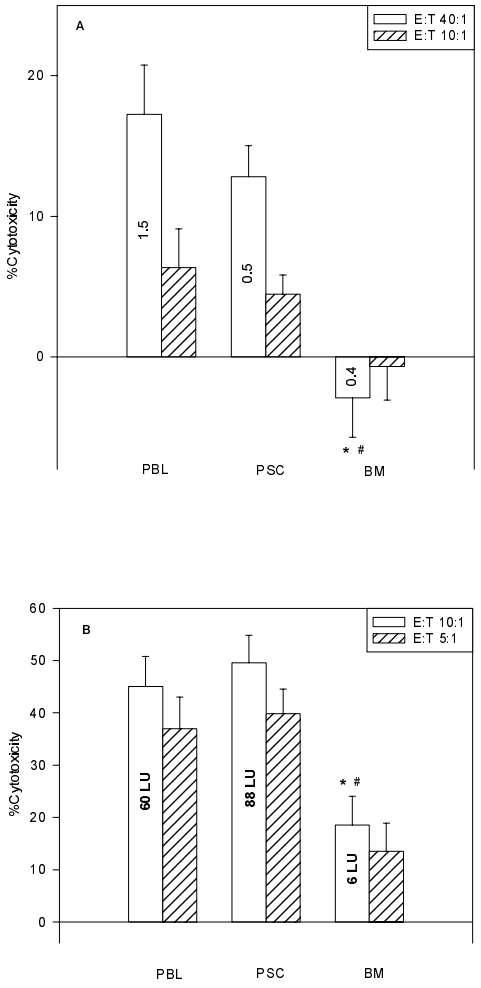

Figure 1. NK cytotoxicity levels against K562 in PBL from normal donors and PSC and BM from NHL patients before in vitro activation with IL-2 (A). NK cytotoxic activity of PBL, PSC and BM cells cultured for 5 days in the presence of 100 U/ml IL-2 (B). Data represent the mean specific lysis ± SE. The lytic unite ($LU_{20}/10^7$ cells) are shown in the bar. *Significant difference compared to PBL ($p<0.001$). #Significant difference compared to PSC ($p<0.001$).

A number of studies have shown a reduced NK cell activity in the PSC of NHL patients (15,26-28). Mazumder *et al.* showed that activated BM cells prior to infusion by incubating the cells with IL-2 results in increased NK cell activity (29,30). In addition, NK cell activity was restored more quickly after PSCT than after autologous BMT (3). Twenty days after ABMT using IL-2 activated BM cells, NK cell activity was normal while NK cell activity

after ABMT requires 30 days to recover (3). The available data suggest that if patients are given IL-2 augmented BM that they may reduce immunosuppression that is normally observed.

The present study showed that the PSC products had higher NK activity than BM products ($p<0.01$), but there was no difference between PBL and PSC. The NK activity of IL-2 co-cultured PSC, BM and PBL was increased 176, 14 and 40 folds compared to those observed prior to IL-2 culture ($p=0.001$, $p<0.001$ and $p<0.001$, respectively). Verma *et al.* also evaluated the effect of IL-2 on normal BM cultures for up to 2 weeks. Their results demonstrated that BM cells had an increased NK cytotoxic activity following *in vitro* culture with IL-2 (31). Thus, PSC products cultured with IL-2 may result in a reduction in PSC contaminating tumor cells compared with BM cells.

In summary, we showed that defective NK cytotoxicity of NHL patients can be repaired in culture of the stem cell product with IL-2. Since such treated effector cells also acquire lytic activity against fresh tumor cells, it is reasonable to suggest that treatment of NHL patients with IL-2 or adoptive transfer of in vitro activated autologous NK cells, may use in the treatment of lymphoma.

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