Protective effects of cytokine combinations against the apoptotic activity of glucocorticoids on CD34⁺ hematopoietic stem/progenitor cells

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Received: 12 February 2018 / Accepted: 9 October 2018 / Published online: 2 January 2019
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Abstract Haematopoietic stem cells can self-renew and produce progenitor cells, which have a high proliferation capacity. Chemotherapeutic drugs are toxic to normal cells as well as cancer cells, and glucocorticoids (GCs), which are essential drugs for many chemotherapeutic protocols, efficiently induce apoptosis not only in malignant cells but also in normal haematopoietic cells. Studies have shown that haematopoietic cytokines can prevent the apoptosis induced by chemotherapy and decrease the toxic effects of these drugs. However, the apoptosis induction mechanism of GCs in CD34⁺ haematopoietic cells and the anti-apoptotic effects of cytokines have

not been well elucidated. In this study, we investigated the apoptotic effects of GCs on CD34⁺, a haematopoietic stem/progenitor cell (HSPC) population, and demonstrated the protective effects of haematopoietic cytokines. We used a cytokine cocktail containing early-acting cytokines, namely, interleukin-3 (IL-3), thrombopoietin, stem cell factor and flt3/flk2 ligand, and dexamethasone and prednisolone were used as GCs. Apoptotic mechanisms were assessed by immunohistochemical staining and quantified using H-scoring. Dexamethasone and prednisolone induced apoptosis in CD34⁺ HSPCs. GC treatment caused a significant increase in apoptotic Fas, caspase-3, cytochrome c and Bax, but a significant decrease in anti-apoptotic Bcl-2. Furthermore, as expected, cytokines caused a significant decrease in all apoptotic markers and a significant increase in Bcl-2. Thus, our findings suggest that CD34⁺ HSPCs are an extremely sensitive target for GCs and that cytokines protect these cells from GC-induced apoptosis.

Keywords CD34⁺ haematopoietic stem/progenitor cells · Glucocorticoids · Cytokines · Apoptosis

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Introduction

Haematopoietic stem cells (HSCs) are defined by their high potential for self-renewal and their ability to

differentiate into all blood cell types (Ogawa 1993; Alenzi et al. 2009). Mature blood cells have a short lifespan, and their continuous replenishment depends directly on HSCs. Thus, HSCs are very important because they maintain lifelong haematopoiesis in organisms. Haematopoietic progenitor cells that cycle rapidly for effective population expansion are also important (Alenzi et al. 2009). Chemotherapy induces apoptosis not only in malignant cells, but also in normal cells such as those involved in haematopoiesis because most chemotherapeutic agents are non-selective and directly target all cells that have a high proliferative potential. Thus, immature haematopoietic cells can suffer from the toxic effects of chemotherapeutic agents, leading to a disruption of haematopoietic regeneration (Zeuner et al. 2003).

Synthetic glucocorticoids (GCs) such as prednisolone and dexamethasone are widely used in cancer therapy. They strongly induce apoptosis in all cells and, owing to this property, they have become essential drugs in all chemotherapeutic protocols (Dowd and Miesfeld 1992; Schwartzman and Cidlowski 1994; Chauhan et al. 1997; Schmidt et al. 1999; Smets et al. 1999; Amsterdam and Sasson 2002; Greenstein et al. 2002). Unfortunately, GCs exert their strong apoptotic effect not only in malignant cells but also in normal haematopoietic cells, such as peripheral blood monocytes, thymocytes, macrophages and lymphocytes (Dowd and Miesfeld 1992; Chauhan et al. 1997; Schmidt et al. 1999; Amsterdam and Sasson 2002; Schmidt et al. 2004).

GCs induce apoptosis by directly regulating the extrinsic and intrinsic apoptotic pathways (Herr et al. 2007), and multiple studies have identified a large number of genes that are responsible for GC-induced apoptosis (Schmidt et al. 2004; Herold et al. 2006). While the role of GCs in apoptosis has been well studied, the effects of cytokines on their mechanisms of action have not been fully elucidated.

The viability and proliferation of haematopoietic cells are strictly dependent on haematopoietic cytokines (Williams et al. 1990), and haematopoietic cells die by apoptosis in the absence of cytokines, whereas malignant cells release cytokines in an autocrine manner (Kinoshita et al. 1995; Lotem and Sachs 1999). Several studies have shown that cytokines promote viability and suppress apoptosis in haematopoietic progenitor cells (Williams et al. 1990; Ikebuchi et al. 1988; Williams and Broxmeyer

1988; Bodine et al. 1991; Itoh et al. 1992; Katayama et al. 1993; Sasaki et al. 1993; Li and Johnson 1994; Jacobsen et al. 1995; Keller et al. 1995; Rasko et al. 1995; Lotem and Sachs 1995; Metcalf 2008; Bordoni et al. 2018). Cytokines can also protect cells from apoptotic death caused by chemotherapeutic agents and can decrease their toxic effects on haematopoietic cells (Griffin and Löwenberg 1986; Lotem and Sachs 1999; Zeuner et al. 2003). Other reports have shown that interleukin-3 (IL-3), thrombopoietin (Tpo), stem cell factor (SCF) and flt3/flk2 ligand (FL) all promote survival and suppress chemotherapy-induced apoptosis in CD34⁺ haematopoietic cells (Williams et al. 1990; Koury and Bondurant 1990; Brandt et al. 1994; Borge et al. 1997; Drouet et al. 1999; Du et al. 2015).

Although the apoptotic effects of GCs on haematopoietic cells have been frequently investigated, no comprehensive studies have been conducted on the apoptotic effects of corticosteroids on the CD34⁺ haematopoietic stem/progenitor cell (HSPC) population or on cytokines' protective anti-apoptotic role in the face of GCs. In this study, we investigated, for the first time, the apoptotic effects of GCs on a CD34⁺ HSPC population isolated from human peripheral blood buffy coat using an immunomagnetic positive selection method and demonstrated the protective effects of haematopoietic cytokines. High concentrations of dexamethasone and prednisolone were used as GCs at concentrations consistent with clinical treatment doses. In order to demonstrate the protective role of cytokines that act early on haematopoiesis, IL-3, Tpo, SCF and FL were used in a pre-treatment cocktail prior to drug application. Immunohistochemical staining was carried out with monoclonal anti-Fas, caspase-3, cytochrome *c*, Bax and Bcl-2 antibodies to determine which molecules were involved in the apoptotic mechanism.

Materials and methods

Enrichment and purification of CD34⁺ HSPCs

A CD34⁺ HSPC population was collected from human peripheral blood buffy coat using an immunomagnetic positive selection method with the Dynal CD34 Human Progenitor Cell Selection Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The buffy coat was

obtained from standard blood collection bags of volunteer blood donors.

The buffy coat was diluted 1:2.5 with phosphate-buffered saline (Zymed, Thermo Fisher Scientific, Waltham, MA, USA) (pH = 7.4) containing 0.1% foetal bovine serum (Gibco, Thermo Fisher Scientific) and 2 mM disodium EDTA (Santa Cruz Biotechnology, Dallas, TX, USA), and peripheral blood mononuclear cells were then isolated by Ficoll-Hypaque (Biocoll, Separating Solution, Biochrom AG, Berlin, Germany) gradient centrifugation. Briefly, peripheral blood mononuclear cells were incubated with Dynabeads (Dyna, Thermo Fisher Scientific) coated with a mouse anti-human CD34 monoclonal antibody for 30 min at 4 °C with a cell-to-bead ratio of 1:1 and using a Dynabeads Mixer (Dyna, Dynabeads MX Mixer Base 159-02, Thermo Fisher Scientific). Rosetted cells were separated using a magnetic particle concentrator (Dyna, Dynamag-2 123.21D, Thermo Fisher Scientific), and nonrosetted cells (CD34[−]) were removed. Captured cells were washed three times to remove non-specifically bound cells. Positively selected CD34⁺ cells were detached from the beads by incubating with an anti-Fab antibody for 45 min at room temperature by mixing according to the manufacturer's instructions. Isolated cells, free of beads, were washed and then counted after trypan blue (Sigma, St. Louis, MO, USA) staining to determine viability (99% of the cells were viable).

The purity of the enriched cells was determined by flow cytometry after labelling with fluorescein isothiocyanate-conjugated mouse anti-human CD34 monoclonal antibody (BioLegend, San Diego, CA, USA) for 30 min at room temperature. The purity of the CD34⁺ cells was between 81% and 93.4% (Fig. 1). CD34⁺ cells were seeded on 24-well cell culture plates (Greiner Bio-One, CELLSTAR Sigma) at 10³ cells/mL in a serum-free medium (Ex-Cell, Bioscience, USA) containing 80 IU/mL penicillin (Icilline, Ulagay, Istanbul, Turkey) and 80 µg/mL streptomycin (Ulagay, Istanbul, Turkey) and cultured at 37 °C in 5% CO₂ air (Nuaire, Caerphilly, UK).

Cytokine and GC treatment

IL-3 (Chemicon, Tokyo, Japan), Tpo (Millipore, Billerica, MA, USA), SCF (Chemicon, Tokyo, Japan) and FL (eBioscience, Thermo Fisher Scientific) were all used at 50 ng/mL (Drouet et al. 1999; Hérodin et al.

2003), and cells were incubated with cytokines for 24 h. Dexamethasone (Dekort, Deva, Istanbul, Turkey) and prednisolone (Prednol-L, Mustafa Nevzat, Istanbul, Turkey) were used at a concentration of 1 mM (Ozbek et al. 1999). After cytokine incubation, test groups were treated with drugs and incubated for a further 24 h.

Control and experimental groups

Two control groups were used. The first was incubated only with a serum-free medium (SFC), whereas the second (CC) was incubated with a cytokine cocktail containing IL-3, SCF, Tpo and FL. Test groups were the dexamethasone group (D), which was incubated with dexamethasone, the prednisolone group (P), which was incubated with prednisolone, the cytokine-dexamethasone group (CD), which was pre-treated with cytokines before dexamethasone treatment, and the cytokine-prednisolone group (CP), which was pre-treated with cytokines before prednisolone treatment.

Immunohistochemical staining

Immunohistochemical staining was conducted using monoclonal antibodies against Fas, caspase-3, cytochrome *c*, Bax and Bcl-2, which were purchased from Santa Cruz Biotechnology, USA. For the immunohistochemical analysis, cells were fixed and dehydrated via a series of increasing alcohol concentrations prior to incubation with anti-caspase antibodies in conjunction with peroxidase-conjugated secondary antibodies (Engin et al. 2009). All sections were examined under a standard light microscope using × 40 objective and 10 × 10 eyepiece incorporating a graticule. For determining degree of peroxidase reaction in CD34⁺ hematopoietic cells, an H-score of ≥ 20 in 10 fields at × 400 magnification was considered positive (Parton et al. 2002) (Fig. 2): (H-score = (% of cells stained at intensity 1 × 1) + (% of cells stained at intensity 2 × 2) + (% of cells stained at intensity 3 × 3)).

Statistical analysis

Results were analysed using GraphPad Prism 5 statistical software. Paired Student's *t* tests and one-sample *t* tests were applied. Values are reported as mean ± standard error of the mean. Control and

Fig. 1 The purity of enriched cells were determined by flow cytometry, after isolation cells were incubated with fluorescein isothiocyanate (FITC) conjugated mouse antihuman CD34 monoclonal antibody. The purity of the CD34⁺ cells was between 81 and 93.4% as seen in sample above. (Control refers to the group incubated only with serum free medium (SFC)). (UL: Upper Left; UR: Upper Right; LL: Lower Left and LR: Lower Right)

Fig. 2 Determining degree of peroxidase reaction in CD34⁺ hematopoietic cells. (++++) cells stained at intensity 3, (++) cells stained at intensity 2, (+) cells stained at intensity 1, (–) no reaction

experimental groups were analysed with a one-sample *t* test. Statistical significance was determined between groups using paired *t* tests. Differences were considered significant when $p < 0.05$.

Results

Immunohistochemistry revealed that dexamethasone and prednisolone treatment increased staining for the apoptotic markers Fas, caspase-3, cytochrome *c* and Bax and decreased staining for the anti-apoptotic Bcl-2, when compared to control groups. Cytokines reduced staining for the apoptotic Fas, caspase-3, cytochrome *c* and Bax and increased staining for anti-apoptotic Bcl-2. The lowest immunoreactivity was observed for Fas and the highest immunoreactivity was observed for caspase-3. After examination, cells were counted and the degree of peroxidase staining was quantified using an H-score (Table 1).

In the absence of cytokines, a significant increase was observed for all apoptotic markers (Fas, caspase-3, cytochrome *c* and Bax) in SFC when compared with CC ($p < 0.05$). By contrast, a significant increase was observed for anti-apoptotic Bcl-2 in CC compared to SFC ($p < 0.05$; Fig. 3). When compared with SFC, both dexamethasone and prednisolone caused a significant increase in apoptotic Fas, caspase-3, cytochrome *c* and Bax in the D and P groups, respectively ($p < 0.05$). However, when compared with CC, there was a significant increase in the CD and CP groups ($p < 0.05$). When cytokines were pre-incubated prior to corticosteroids, a significant

Table 1 Expression levels of Fas, caspase 3, cytochrome c, Bax, Bcl2 proteins in experiment and control groups

Groups	Mean $\pm$ SD				
	Fas	Caspase 3	Cytochrome c	Bax	Bcl 2
Serum free control (SFC)	147.6 $\pm$ 3.208	204.0 $\pm$ 6.263	181.6 $\pm$ 2.816	176.3 $\pm$ 2.982	160.4 $\pm$ 6.879
Cytokine control (CC)	82.50 $\pm$ 2.851	155.6 $\pm$ 4.315	135.0 $\pm$ 12.42	130.4 $\pm$ 7.855	184.4 $\pm$ 9.198
Dexamethasone (D)	185.1 $\pm$ 8.649	227.5 $\pm$ 6.346	194.5 $\pm$ 2.326	185.8 $\pm$ 0.6807	136.2 $\pm$ 5.150
Cytokine-dexamethasone (CD)	83.63 $\pm$ 3.868	167.8 $\pm$ 4.646	159.8 $\pm$ 4.620	136.2 $\pm$ 2.250	166.8 $\pm$ 6.476
Prednisolone (P)	177.9 $\pm$ 3.119	229.1 $\pm$ 5.805	208.5 $\pm$ 5.197	187.0 $\pm$ 6.671	142.1 $\pm$ 5.801
Cytokine-prednisolone (CP)	112.4 $\pm$ 8.528	173.1 $\pm$ 5.937	170.9 $\pm$ 3.928	140.5 $\pm$ 7.030	175.8 $\pm$ 3.819

Values are mean $\pm$ SD for five individual experiments. Determined by H-score

Fig. 3 Peroxidase reaction levels calculated using H-score between serum free medium control and cytokine control that including IL3, SCF, Tpo and FL. The results represent the means $\pm$ SD of five individual experiments performed with cells from different donors. * $p < 0.05$

decrease in the CD and CP groups was observed compared to the D and P groups ($p < 0.05$; Fig. 4). A significant reduction was observed in the D and P groups for anti-apoptotic Bcl-2 when compared with SFC ($p < 0.05$), whereas a significant decrease was observed in the CD and CP groups when compared with CC ($p < 0.05$). In contrast, cytokine pre-incubation caused a significant increase in Bcl-2 in the CD and CP groups compared to the D and P groups ($p < 0.05$; Fig. 5).

Discussion

GCs are used as essential drugs in most treatment protocols for various haematological malignancies, such as lymphoma, leukaemia and myeloma, because of their strong apoptotic and cytotoxic effects on malignant haematopoietic cells (Dowd and Miesfeld 1992; Schwartzman and Cidrowski 1994; Chauhan et al. 1997; Schmidt et al. 1999; Smets et al. 1999; Amsterdam and Sasson 2002; Greenstein et al. 2002). However, GCs induce apoptosis in healthy cells of haematological lineages, so the haematopoietic system can be destroyed by treatment with these agents (Dowd and Miesfeld 1992; Chauhan et al. 1997; Schmidt et al. 1999; Amsterdam and Sasson 2002). GC-induced apoptosis has been well studied, but the role of cytokines in the apoptotic mechanisms involved in this process is not yet clear. In particular, there have been no comprehensive studies regarding the apoptotic effects of corticosteroids on pure CD34⁺ HSPC populations or on the protective role of cytokines with respect to the apoptosis caused by GCs. In this study, we demonstrated, for the first time, the apoptotic effects of high GC concentrations on a CD34⁺ HSPC population and the protective effects of low concentrations of haematopoietic cytokines.

It is known that corticosteroids directly activate apoptotic processes by regulating components of the extrinsic pathway, such as Fas and tumour necrosis factor receptors, and the intrinsic mitochondrial pathway, such as Bcl-2, Bax and cytochrome *c* (Herr et al. 2007). In this study, when compared with control groups, corticosteroid treatment resulted in a significant increase in apoptotic Fas, caspase-3, Bax and cytochrome *c* levels ($p < 0.05$) and a significant

Fig. 4 Peroxidase reaction levels calculated using H-score in cells stained with apoptotic markers. **a** Reaction levels for Fas. **b** Reaction levels for Caspase 3. **c** Reaction levels for cytochrome c. **d** Reaction levels for Bax show expression levels of apoptotic proteins. (SFC) Serum free control, (CC) Cytokine control, (CD) Cytokine-dexamethasone, (D) Dexamethasone,

(CP) Cytokine-prednisolone, (P) Prednisolone represent experiment and control groups. The results represent the means $\pm$ SD of five individual experiments performed with cells from different donors. *, **, ***, **** $p < 0.05$. More asterisk were used for comparison between different groups, but statistically significant was same value

decrease in anti-apoptotic Bcl-2 level ($p < 0.05$; Fig. 4).

Studies using mouse thymocytes have shown that the Fas-induced apoptosis pathway functions in GC-mediated apoptosis (D'Adamio et al. 1997; Ashwell et al. 2000). In this study, GCs applied at high concentrations (1 mM) caused a significant increase in Fas in CD34⁺ HSPCs because increases were observed in the D and P groups when compared with the SFC group. In addition, a significant increase was observed in the CD and CP groups compared with the CC group. This result indicates that the Fas ligand is involved in GC-induced apoptosis in CD34⁺ HSPCs. It is known that CD34⁺ haematopoietic cells do not

express Fas until they differentiate (Maciejewski et al. 1995; Nagafuji et al. 1995). Additionally, Kim et al. (2002) showed that CD34⁺ HSCs in human placental/umbilical cord blood and adult mobilised blood (PBSC) express high levels of Fas inhibitor. These observations are consistent with our findings.

Several reports have shown that caspase-3, as the key effector caspase in corticosteroid-mediated apoptosis and strong induction of caspase-3 in GC-mediated apoptosis, has been demonstrated in leukaemic cells (Chandra et al. 1997; Robertson et al. 1997; McColl et al. 1998; Kofler 2000). Dexamethasone-induced apoptosis of human B-cell leukaemia cell lines has been shown to involve caspase-3 activation

Fig. 5 Peroxidase reaction levels calculated using H-score for antiapoptotic Bcl-2. (SFC) Serum free control, (CC) Cytokine control, (CD) Cytokine-dexamethasone, (D) Dexamethasone, (CP) Cytokine-prednisolone, (P) Prednisolone. The results represent the means $\pm$ SD of five individual experiments. *, **, ***, **** $p < 0.05$. More asterisk were used for comparison different groups, but statistically significant was same value

in many in vitro studies (Miyashita et al. 1998; Liu et al. 2014). Additionally, it has been shown that dexamethasone causes caspase-3 activation in mouse lymphoma cells (McColl et al. 1998). In this study, both D and P groups exhibited a significant increase in caspase-3 compared to the SFC group. Furthermore, a significant increase in caspase-3 was observed in the CD and CP groups when compared with the CC group, and the highest immunoreactivity was observed for caspase-3. It is known that effector caspases are activated by two apoptotic pathways: the extrinsic death receptor and the intrinsic mitochondrial pathways (Nunez et al. 1998). However, Steidl et al. (2002) measured gene expression profiles in human bone marrow and peripheral CD34⁺ haematopoietic cells, and they have shown that caspase-3 is expressed at a higher level in peripheral blood CD34⁺ haematopoietic cells. These results are in agreement with ours with respect to caspase-3.

GCs exert their apoptotic effects through interaction with the GC receptor (GR). It is known that the GR is a nuclear receptor, although a cell-membrane-associated receptor has been described (Scheller et al. 2000; Moutsatsou et al. 2001). In addition, GR has been shown to be located within the mitochondria of some cell types because mitochondrial GR translocation occurs in GC-sensitive cells upon dexamethasone induction (Sionov et al. 2006). Furthermore, several studies have shown that GCs in high doses interact

with membranes and alter their physicochemical properties (Buttgereit and Scheffold 2002; Buttgereit et al. 2004). In this study, high doses of GCs may have resulted in interaction with mitochondrial membranes disrupted of membrane potential and released cytochrome *c* into the cytoplasm. The intrinsic mitochondrial apoptosis pathway seems to be important for GC-induced apoptosis (Tissing et al. 2003; Heidari et al. 2012; Li et al. 2016). It has been shown by several studies that dexamethasone treatment induces loss of mitochondrial membrane potential in thymocytes and leukaemic T-cells (Castedo et al. 1995; Camilleri-Broët et al. 1998; Heidari et al. 2012). In addition, pro-apoptotic and anti-apoptotic members of the Bcl-2 family play an important role in GC-induced apoptosis. GCs induce pro-apoptotic members and suppress anti-apoptotic members of the Bcl-2 family (Han et al. 2001; Chauhan et al. 2002; Casale et al. 2003; Wang et al. 2003; Spijkers-Hagelstein et al. 2014; Moriishi and Komori 2014). In vitro studies using leukaemia and lymphoma cell lines have shown that dexamethasone treatment induces upregulation of Bax and downregulation of Bcl-2 (Lotem and Sachs 1995; Schwarze and Hawley 1995; McConkey et al. 1996). Furthermore, it has been demonstrated that dexamethasone causes cytochrome *c* release and caspase-3 activation in multiple myeloma cells (Chauhan et al. 1997). In this study, dexamethasone and prednisolone caused a significant increase in the Bax and cytochrome *c* levels and a significant decrease in the Bcl-2 level when compared with the SFC group, and similar results were obtained in the CD and CP groups. Thus, it appears that GCs induce the mitochondrial apoptotic pathway in CD34⁺ HSPCs and so regulate the Bax/Bcl-2 molecular rheostat.

Human CD34⁺ haematopoietic cells represent a heterogeneous population including primitive stem cells, primitive progenitors, multipotent progenitors and numerous lineage-committed progenitors at different developmental stages (Furukawa 1998; Steidl et al. 2002; Kondo et al. 2003; Bonde et al. 2004; Majka et al. 2005). In this study, GCs may have induced apoptosis leading to cell cycle arrest in self-renewing stem cells in the G1 phase, in actively cycling primitive progenitors and in rapidly cycling committed progenitors. GCs induce apoptosis through at least two separate pathways in proliferating or non-proliferating cells. It is known that CD34⁺ cells residing in the bone marrow cycle more rapidly than

those in the circulation, which consist of a higher number of quiescent stem and progenitor cells. Many groups have demonstrated that peripheral blood stem cells (PBSCs) have a markedly lower cell cycle activity than bone marrow CD34⁺ cells and that PBSCs are in the G0/G1 phase of the cell cycle (Ng et al. 2004). As a result, it may be that, in our study, GCs induced apoptosis in a cell-cycle-independent manner in quiescent stem and progenitor cells in the G0 phase. In addition, the GC concentrations that we used in this study were higher than those used for in vitro expansion and differentiation of stem cells (0.1–1 µM; Grafte-Faure et al. 1999).

We then used low doses of early-acting cytokines such as IL-3, Tpo, SCF and FL to protect against the toxic effects of GC and to maintain the cycle. These different cytokines have similar and sometimes overlapping functions when used in combination. These similar functions are mediated by a family of cytokine receptors (Lotem and Sachs 2002) because cytokine receptors have partly homologous structures (Brach and Hermann 1991; Lotem and Sachs 2002). M-CSF, SCF and FL receptors share a related intra-cellular tyrosine kinase domain, whereas GM-CSF, G-CSF, IL-3 and Tpo receptors recruit src-related cytoplasmic tyrosine kinases and JAK protein kinases to transmit their intra-cellular signals (Lotem and Sachs 2002). Thus, in our study, cytokines that exhibit anti-apoptotic effects were used in a combination. Cytokines are known to affect haematopoietic cells at different stages (Metcalf 2008). In this respect, cytokines may be divided into categories according to the differentiation stage of the cells that they regulate. Because of this, we used early-acting cytokines that support proliferation and maturation of HSCs and primitive progenitors. Hence, cytokines promote viability and suppress apoptosis in haematopoietic cells, and in the absence of cytokines, haematopoietic cells die by apoptosis (Williams et al. 1990; Kinoshita et al. 1995). It has been shown that a combination of IL-3, SCF, Tpo and FL promotes survival and suppresses natural and chemotherapy-induced apoptosis in CD34⁺ haematopoietic cells by increasing the expression of apoptosis-suppressing genes and decreasing the expression of apoptosis-inducing genes (Williams et al. 1990; Koury and Bondurant et al. 1990; Brandt et al. 1994; Borge et al. 1997; Drouet et al. 1999). Sigurdsson et al. (2004) have shown that a

combination of Tpo and FL suppresses apoptosis in bone marrow CD34⁺ cells in a cell culture.

Several studies have demonstrated that IL-3 induces Bcl-2 gene expression (Deng and Podack 1993; Kinoshita et al. 1995). In addition, EPO, IL-3 and SCF suppress apoptosis, induce the upregulation of Bcl-2 and protect the mitochondrial membrane potential in erythroid progenitor cells (Wang et al. 2007).

Another study showed that a combination of SCF, FL, Tpo and IL-3 decreases Fas expression and increases Bcl-2 expression in peripheral blood CD34⁺ cells (Drouet et al. 1999). In our study, the addition of IL-3, Tpo, SCF and FL to the culture medium resulted in a significant decrease in the apoptotic caspase-3, Fas, cytochrome *c* and Bax levels and a significant increase in the anti-apoptotic Bcl-2 level in all groups at low concentrations. Because in some studies Tpo and FL are used at high concentrations (100 ng/mL) against the harmful effects of radiation and drugs. (Ahmed et al. 2004; Dooley et al. 2004; Choi et al. 2010).

Several studies have demonstrated that SCF decreases the caspase-3 activation induced by chemotherapeutic agents and increases the anti-apoptotic Bcl-2 expression. In addition, IL-3 protects haematopoietic cells from apoptosis induced by chemotherapeutic agents (Zeuner et al. 2003). Drouet et al. (1999) showed that a combination of SCF, FL, Tpo and IL-3 decreases radiation-induced Fas expression and induces a high level of Bcl-2 expression in CD34⁺ HSCs. In our experiments, when IL-3, Tpo, SCF and FL were added prior to corticosteroids, the anti-apoptotic Bcl-2 levels exhibited a significant increase ($p < 0.05$) and apoptotic caspase-3, Fas and cytochrome *c* levels decreased significantly ($p < 0.05$) when compared with the CC group (Fig. 5).

Conclusion

In clinics, high doses of GCs are simultaneously administered in haematological malignancies and solid tumour chemotherapies. In addition, due to their anti-inflammatory and anti-allergenic properties, long-term high doses of GCs are used in the treatment of many diseases. Considering their haematopoietic toxicity, GCs should not be used lightly. Thus, identifying the precise mechanisms of their action

will provide significant insights into their cytotoxic effects and allow the development of novel strategies that are more selective in their action. The results presented in this study identify cytokines as protective factors for CD34⁺ HSPCs during treatment with high doses of GCs and may stimulate future investigations into the use of these cytokines in the supportive care of patients with cancer for recovering the blood cell production functionality.

We therefore suggest that cytokine treatment may be useful in the clinical practice in order to minimise haematopoietic toxicity and to protect CD34⁺ HSCs from damage from high doses of corticosteroids.

Acknowledgements The authors thank Prof. Günnur Deniz and Mr. Abdullah Yılmaz, Department of Immunology, Istanbul University, Institute of Experimental Medicine for their generous help with providing facilities deals with flow cytometric studies. Also we wish to express our gratitude to staff of the Istanbul Medical Faculty Blood Bank for generous help with providing human peripheral blood buffy coats. The study was supported by the Research Fund of Istanbul University. Project No: 2980.

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