The influence of antisense oligonucleotides against STAT5 on the regulation of normal haematopoiesis in a bone marrow model

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Abstract. Cytokines and growth factors that take part in the regulation of haematopoietic cell development activate many signalling pathways in target cells. The STAT5 (signal transducers and activators of transcription) proteins are members of a family of signal transducers and activators of transcription that can be activated after cytokine stimulation. Their binding to promoters of different genes influences cell proliferation, differentiation and survival. It is suggested that they play an important role in haematopoiesis, however, the question of the real function of STAT5 proteins requires further examination. The aim of our study was to investigate the role of STAT5 in the proliferation and apoptosis of normal haematopoietic bone marrow cells derived from heparinized cadaveric organ donors (HCOD). We applied antisense oligodeoxynucleotides (ODNs) to block STAT5A and STAT5B at the mRNA level and the reverse transcription polymerase chain reaction method to study STAT5 mRNA expression in the cells after incubation with ODNs. Moreover, we performed Western blot analysis of the STAT5A protein after exposure to antisense STAT5A. We analysed the clonogenicity of the colony-forming unit of granulocytes–macrophages and the burst-forming unit of erythrocytes in methylcellulose cultures according to the type and the dose of ODNs. We also examined apoptosis induced in bone marrow mononuclear and CD34⁺ cells by employing annexin V staining and the TUNEL method using flow cytometry (FACScan). We found that the perturbation of STAT5 expression decreased the clonogenicity of bone marrow haematopoietic cells. However, we did not observe any significant increase in the percentage of apoptotic cells after incubation with antisense ODNs. It was concluded that the STAT5 proteins play a significant role in the proliferation of human bone marrow cells harvested from HCOD. These proteins might be critical in the regulation of haematopoiesis, especially under stress conditions.

INTRODUCTION

Haematopoiesis is regulated by many cytokines, growth factors and hormones, for example stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), IL-5, granulocyte-colony stimulating factor, thrombopoietin and erythropoietin (EPO). Following their binding to receptors on the cell surface different signalling pathways are activated (Leonard & Lin 2000). Some of the signalling proteins phosphorylated after cytokine stimulation are members of the signal transducers and activators of transcription (STAT) family (Darnell 1997; Aaronson & Horvath 2002). The STAT family was discovered and described in 1992 and the structure and mechanism of activation of these proteins is well known (Ihle 1996; Weber-Nordt *et al.* 1998). Seven STAT proteins have been identified: STAT1–4, STAT6 and two very similar proteins STAT5A and STAT5B. The STAT5 proteins are activated by many of the same cytokines; however, they also have different functions in different types of cells (Akira 1999; Grimley *et al.* 1999). STAT5s have been proved to play an important role in many of the physiopathological processes related to the immune responses; cell growth, cell cycle regulation and cell transformation (Weber-Nordt *et al.* 1998; Akira 1999; Bowman *et al.* 2000; Bromberg & Darnell 2000). *In vivo* experiments on knock-out mice and *in vitro* studies in established cell lines have suggested a role in haematopoiesis (Akira 1999; Ilaria *et al.* 1999; Kieslinger *et al.* 2000). The STAT5s have been shown to be necessary for erythroid cell development of mouse embryos and neonates (Socolovsky *et al.* 1999). Many experiments have been performed on adult STAT5A^{-/-}B^{-/-} mice, and there are many contradictory reports on the role of STAT5 proteins in the haematopoiesis of adult individuals. Adult STAT5-deficient mice were reported to have a normal haematocrit, and normal numbers of red cells, platelets, neutrophils and monocytes, with only lymphocytes being decreased in number (Teglund *et al.* 1998). On the other hand, studies on the erythropoiesis of adult STAT5A^{-/-}B^{-/-} mice showed that approximately half the mice suffered from persistent anaemia (Socolovsky *et al.* 2001). In addition, adult STAT5A^{-/-}B^{-/-} mice with normal haematocrit were deficient in generating a high erythropoietic rate in response to erythropoietic stress (Socolovsky *et al.* 2001), and STAT5s were suggested to regulate early erythroblast survival (Socolovsky *et al.* 2001). Moreover, studies on the repopulating activity of haematopoietic stem cells from adult STAT5A^{-/-}B^{-/-} mice have been performed (Bradley, Hawley & Bunting 2002; Bunting *et al.* 2002; Snow *et al.* 2002). Stem-cell multilineage repopulating potential was shown to be highly STAT5 dependent and STAT5A^{-/-}B^{-/-} haematopoietic stem cells transplanted to lethally irradiated recipient mice had markedly decreased reconstituting capacity. Surprisingly, the number of haematopoietic stem cells was not different between wild-type and STAT5A^{-/-}B^{-/-} bone marrows but the post-haematopoietic stem cell progenitor populations were decreased in STAT5A/5B-deficient mice (Bradley *et al.* 2002; Bunting *et al.* 2002; Snow *et al.* 2002). The influence of STAT5 on haematopoiesis requires further study.

It has been previously demonstrated that the STAT5 proteins do not play a significant role in the regulation of proliferation and survival of normal human haematopoietic cells derived from cord blood (Bařkiewicz-Masiuk *et al.* 2003a). In this study we have attempted to determine whether STAT5s are critical in the growth of adult human haematopoietic cells. Human bone marrow haematopoietic cells obtained from heparinized cadaveric organ donors (HCOD) were used because HCOD have been reported to be a potential source of haematopoietic cells for transplantation and gene therapy (Machaliński *et al.* 2001b). Antisense oligonucleotides against the STAT5A as well as the STAT5B mRNA were employed to explore if these proteins have different functional activities in haematopoietic cell proliferation and apoptosis. The clonogenicity of bone marrow haematopoietic cells was analysed in methylcellulose cultures according to the dose and the type of antisense oligodeoxynucleotide (ODN) used. Apoptosis levels in ODN-treated cells were also tested.

MATERIALS AND METHODS

Cells

Bone marrow samples (60–160 ml) were harvested from the pelvic bones of 12 brain-dead adult HCOD (seven males and five females) before disconnecting the donor from the respirator (Machaliński *et al.* 2001b, 2003). The median age of the donors was 44.1 ± 13.2 years. The cause of death in five cases was a complication of mechanical trauma and in seven cases was the result of bleeding into the central nervous system. The donors had not suffered from haematological diseases. The average time-lapse between notification of brain death and bone marrow collection was 6 ± 1 h and the procedure had been approved by the local ethical committee. In addition, in every case the donors' families gave special permission.

Bone marrow cells were harvested with Yamshida's needles (Manan Medical Products, IL, USA) and were resuspended in the collecting medium [Iscove's modified Dulbecco's medium (IMDM); Gibco, USA] supplemented with heparin (20 U/ml, Gibco, Carlsbad, CA, USA), 5% bovine calf serum (Hyclone Laboratories Inc., Logan, UT, USA), penicillin–streptomycin, and 2 mmol/l L-glutamine (Gibco) (Machaliński *et al.* 2001b). Normal light-density mononuclear cells (MNC) were depleted of adherent cells and T lymphocytes ($A-T^-$ MNC) as described previously (Ratajczak *et al.* 1992; Machaliński *et al.* 1999). Their viability was assessed by a trypan blue exclusion test. The cells were counted using a haemocytometer and subsequently used for further experiments.

CD34⁺ cell isolation

$A-T^-$ MNC were enriched for CD34⁺ cells in six of the donor samples by an immunoaffinity selection with a commercially available CD34⁺ isolation MiniMACS kit (Miltenyi Biotec Inc., Auburn, CA, USA) according to the manufacturer's protocol. Briefly, bone marrow MNC were resuspended at 10^8 per 300 μ l in cold PBE buffer [phosphate buffered saline (PBS), 0.5% bovine serum albumin, 5 mM ethylenediaminetetraacetic acid]. The blocking reagent (100 μ l/ 10^8 cells) and the antibody reagent (100 μ l/ 10^8 cells) from the CD34⁺ isolation kit were added simultaneously to the cell suspension, mixed well and incubated for 30 min at 4 °C. After incubation, the cells were washed and re-suspended in 400 μ l/ 10^8 cells PBE. Then, 100 μ l/ 10^8 cells of microbead-conjugated antibodies was added to the cells, mixed well and incubated for 30 min at 4 °C. Finally, the antibody-labelled population was isolated by employing magnetic columns in a two-round procedure. The purity of the isolated bone marrow CD34⁺ cells was greater than 95%, as determined by flow cytometry analysis. The collected cells were used for further studies (Caldenhoven *et al.* 1998; Majka *et al.* 2000; Buitenhuis *et al.* 2003).

ODNs

ODNs were supplied by MWGBiotech AG (Ebersburg, Germany) as sterile sodium salt, endotoxin-free, lyophilized powders and were re-dissolved in IMDM before use. Compounds were fully phosphorothioated, 24 nucleotides in length, and targeted to codons 1–8 of the *STAT5* mRNA. The ODN sequences employed [antisense (AS) *STAT5A* and *STAT5B*; sense; and scrambled] were as previously described. The sense and scrambled ODN sequences were utilized as an additional control (Baśkiewicz-Masiuk *et al.* 2003a,b).

ODN exposure protocol

Briefly, bone marrow $A-T^-$ MNC or CD34⁺ cells were incubated in 1 ml of PBS containing 10% bovine calf serum for 24 h with AS *STAT5A*, AS *STAT5B*, AS *STAT5A* + B; sense or scrambled at low (L) or high (H) dose (low dose, 75 μ g/ml, high dose, 150 μ g/ml final concentration)

(Majka *et al.* 2000; Luger *et al.* 2002). In the case of the cells incubated with AS STAT5A + B the final concentration of each antisense ODN was 150 µg/ml. After incubation, the cells were washed twice in IMDM and subsequently their viability was assessed by a trypan blue exclusion test. Next, the cells were plated in methylcellulose cultures and the number of cells that were undergoing apoptosis was assessed by employing two different assays at the same time (annexin V/propidium iodide staining and the TUNEL method). Total RNA and protein were extracted from a certain number of the cells for further analysis. Control groups contained cells manipulated in an identical manner but without exposure to ODNs.

STAT5 expression at the mRNA level

After incubation with ODNs, total RNA was isolated from 1×10^6 bone marrow MNC of each donor sample using an RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. STAT5A and STAT5B levels were assessed by a semiquantitative polymerase chain reaction (PCR) as described (Majka *et al.* 2000; Gesbert & Griffin 2000). β -Actin levels were used as an internal control; 1 µg of total RNA was reverse-transcribed to cDNA with Moloney's murine leukaemia virus reverse transcriptase (Promega, Madison, WI, USA). For PCR, cDNA was amplified using β -actin primers (forward: 5'-CCTAAGGCCAACCGTGAAAAG-3'; reverse: 5'-TCTTC-ATGGTGCTAGGAGCCA-3'), STAT5A primers (forward: 5'-GCCGGCTGTGTATGGTCTAT-3'; reverse: 5'-AAGTAGTGCCGGACCTCGAT-3'), STAT5B primers (forward: 5'-GTAAACCATGGCTGTGTGGA-3'; reverse: 5'-AAATAATGCCGCACCTCAAT-3') in a total volume of 50 µl using 2 U *Taq* polymerase (Baśkiewicz-Masiuk *et al.* 2003a,b). The amplification was performed for 25 cycles with an initial hot start at 92 °C for 2 min, followed by 45 s of denaturation at 94 °C, 45 s of annealing at 55 °C, 1 min of extension at 72 °C, and a final extension period of 5 min; 15-µl aliquots were run on a 1.5% agarose gel. The expected sizes of the amplicons were 283 base pairs for STAT5A, and 108 base pairs for STAT5B.

STAT5 expression at the protein level

Western blots were performed using extracts prepared from 1×10^6 bone marrow MNC after a 24-h incubation with ODNs. The cells were lysed for 10 min on ice in M-Per lysing buffer (Pierce, Rockford, IL, USA) containing protease inhibitors (Sigma, St. Louis, MO, USA). Subsequently, the extracted proteins were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis, and the fractionated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). The STAT5A protein was detected using mouse monoclonal antibody (clone 51; Transduction Laboratories, San Jose, CA, USA; gift from Prof. M. Z. Ratajczak from James Graham Brown Cancer Center, University of Louisville, USA) with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin as a secondary antibody (Santa Cruz Biotech, Santa Cruz, CA, USA). The membranes were developed with an enhanced chemiluminescence reagent (Amersham Life Sciences, UK) and subsequently dried and exposed to film (HyperFilm, Amersham Life Sciences, Buckinghamshire, UK) (Ratajczak *et al.* 2003). This procedure was performed three times.

Cell cultures in methylcellulose medium

Bone marrow A-T⁻ MNC (5×10^4) or bone marrow CD34⁺ cells (2×10^4) were resuspended in 0.4 ml IMDM and mixed with 1.8 ml methylcellulose medium MethoCult HCC-4230 (StemCell Technologies Inc., Vancouver, Canada) supplemented with L-glutamine (0.125 mM). Appropriate growth factors were added to the mixture, which was then transferred to 3.5-cm diameter plastic Petri dishes (SARSTED Inc., Newton, NC, USA) and incubated (37 °C, 95% humidity, 5% CO₂) for a time appropriate for the formation of the particular colonies. The growth factors

employed for colony stimulation were as follows: IL-3 (20 U/mL) plus GM-CSF (5 ng/ml) (R & D Systems, Minneapolis, MN, USA) were used for granulocyte-macrophage colony-forming units (CFU-GM); and EPO (5 U/mL), SCF (10 ng/mL) plus IL-3 (20 U/mL) (R & D Systems, USA) were used for erythrocyte burst-forming units (BFU-E). Recombinant human growth factors were employed in all the experiments. The colonies were counted with an inverted microscope (Olympus CR40, Olympus, Tokyo, Japan) on day 11 for CFU-GM and on day 14 for BFU-E. Cultures were performed in quadruplicate. Results were expressed as a percentage of the control values (100%) (Machaliński *et al.* 1999).

Apoptosis assays

Combined annexin V-propidium iodide staining The level of apoptosis was assessed on live MNC and CD34⁺ cells after a 24-h incubation with the ODNs using annexin V-fluorescein isothiocyanate (FITC; BD Biosciences Pharmingen, San Jose, CA, USA) following the manufacturer's specifications. Binding of FITC-conjugated annexin V and propidium iodide (PI) was analysed by flow cytometry (FACScan, BD, USA) (Koopman *et al.* 1994; Gesbert & Griffin 2000; Kieslinger *et al.* 2000).

TUNEL method In addition, apoptosis was also quantified on fixed CD34⁺ cells after a 24-h incubation with the ODNs by using terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL) assays (APO-DIRECT, BD Biosciences Pharmingen, USA) following the manufacturer's instructions. The labelled DNA breaks with FITC-dUTP were visualized on a FACScan flow cytometry (Moulian *et al.* 1999).

Statistical analysis

The arithmetical means and standard deviations were calculated on an IBM computer using MS EXCEL vs. 97. Cells were cultured in quadruplicate at each point. The data were analysed using the Kruskal-Wallis test. The values showing significant differences in the Kruskal-Wallis test were next analysed using the *U* Mann-Whitney test. Statistical significance was defined as $P < 0.05$.

RESULTS

Analysis of STAT5 expression at the mRNA and the protein levels in bone marrow MNC and CD34⁺ cells after incubation with ODNs

It has been previously demonstrated that the ODNs against STAT5 employed in the study specifically suppress their respective targets in haematopoietic cells at the mRNA level (Baśkiewicz-Masiuk *et al.* 2003a,b). Nonetheless, activity of the STAT5-targeted ODNs has been confirmed in human bone marrow MNC. The study was performed on each sample of bone marrow cells. Each of the 12 samples evaluated for ODN-sensitivity demonstrated sequence-specific sensitivity to the anti-STAT5 ODNs. Figure 1 shows that AS STAT5 efficiently inhibited the expression of *STAT5* at the mRNA level after the 24-h incubation. The results observed on bone marrow MNC were confirmed using CD34⁺ cells (Fig. 2). It has also been demonstrated that the anti-STAT5A ODN did not decrease *STAT5B* gene expression nor did AS STAT5B influence *STAT5A* gene expression (Fig. 2).

Sequence-specific sensitivity to the STAT5 antisense product has also been proven by Western blot analysis. Figure 3 shows that AS STAT5A caused suppression of STAT5A protein synthesis in the treated cells after the 24-h exposure. Equivalent results were obtained three times.

Figure 1. Example of *STAT5A* and *STAT5B* mRNA expression in human bone marrow MNC after 24-h incubation with ODNs. RT-PCR products separated on a gel: M, molecular weight DNA marker; lane 1, control cells not exposed to ODNs – β -actin (control of mRNA integrity); lane 2, control cells not exposed to ODNs – *STAT5A*; lane 3, cells exposed to AS *STAT5A* (150 μ g/ml) – β -actin; lane 4, cells exposed to AS *STAT5A* (150 μ g/ml) – *STAT5A*; lane 5, control cells not exposed to ODNs – *STAT5B*; lane 6, cells exposed to AS *STAT5B* (150 μ g/ml) – *STAT5B*.

Figure 2. Example of the analysis of *STAT5A* and *STAT5B* mRNA expression in the bone marrow CD34⁺ cells after a 24-h incubation with ODNs. RT-PCR products separated on a gel: M, molecular weight DNA marker; lane 1, control cells not exposed to ODNs – *STAT5A*; lane 2, cells exposed to AS *STAT5A* (150 μ g/ml) – *STAT5A*; lane 3, cells exposed to AS *STAT5B* (150 μ g/ml) – *STAT5A*; lane 4, cells exposed to scrambled ODNs (150 μ g/ml) – *STAT5A*; lane 5, control cells not exposed to ODNs – *STAT5B*; lane 6, cells exposed to AS *STAT5A* (150 μ g/ml) – *STAT5B*; lane 7, cells exposed to AS *STAT5B* (150 μ g/ml) – *STAT5B*; lane 8, cells exposed to scrambled ODNs (150 μ g/ml) – *STAT5B*.

Figure 3. Analysis of the STAT5A protein in human bone marrow MNC after a 24-h incubation with ODNs by Western blot. Lane 1, control cells not exposed to ODNs (STAT5A – 96 kDa); lane 2, cells exposed to AS STAT5A (150 µg/ml); lane 3, cells exposed to sense ODNs (150 µg/ml); lane 4 – cells exposed to scrambled ODNs (150 µg/ml).

Figure 4. The influence of ODNs on the clonogenicity of human bone marrow CFU-GM ($n = 12$) after a 24-h incubation ($x \pm SD$) (* $P < 0.05$): C, control cells not exposed to ODNs; S-L, cells exposed to sense ODNs (75 µg/ml); S-H, cells exposed to sense ODNs (150 µg/ml); AS STAT5A-L, cells exposed to antisense STAT5A ODNs (75 µg/ml); AS STAT5A-H, cells exposed to antisense STAT5A ODNs (150 µg/ml); AS STAT5B-H, cells exposed to antisense STAT5B ODNs (150 µg/ml); AS STAT5A + B-H, cells exposed to antisense STAT5A and STAT5B ODNs (150 µg/ml); SCR-L, cells exposed to scrambled ODNs (75 µg/ml); SCR-H, cells exposed to scrambled ODNs (150 µg/ml). Results are expressed as a percentage of the control values (100%).

The influence of the ODNs on the clonogenicity of human bone marrow haematopoietic cells

The clonogenic assays in methylcellulose medium were performed to investigate the role of STAT5 in the regulation of bone marrow cell proliferation and differentiation. Both low and high doses of the ODNs were used in bone marrow MNC. It has been observed that the growth of the CFU-GM (myeloid) and the BFU-E (erythroid) colonies decreased after the 24-h incubation with antisense oligonucleotides (Figs 4 and 5). However, in the case of CFU-GM, the differences

Figure 5. The influence of ODNs on the clonogenicity of human bone marrow BFU-E ($n = 12$) after a 24-h incubation ($x \pm SD$) (* $P < 0.05$; ** $P < 0.001$): C, control cells not exposed to ODNs; S-L, cells exposed to sense oligonucleotides (75 $\mu\text{g/ml}$); S-H, cells exposed to sense ODNs (150 $\mu\text{g/ml}$); AS STAT5A-L, cells exposed to antisense STAT5A ODNs (75 $\mu\text{g/ml}$); AS STAT5A-H, cells exposed to antisense STAT5A ODNs (150 $\mu\text{g/ml}$); AS STAT5B-H, cells exposed to antisense STAT5B ODNs (150 $\mu\text{g/ml}$); AS STAT5A + B-H, cells exposed to antisense STAT5A and STAT5B ODNs (150 $\mu\text{g/ml}$); SCR-L, cells exposed to scrambled ODNs (75 $\mu\text{g/ml}$); SCR-H, cells exposed to scrambled ODNs (150 $\mu\text{g/ml}$). Results are expressed as a percentage of the control values (100%).

between the group of control cells and the cells incubated with AS STAT5B were not statistically significant ($P > 0.05$). Differences in the growth of bone marrow MNC after exposure to AS STAT5A in the low and high doses were not statistically significant. Moreover, the clonogenic potential of bone marrow MNC after incubation with mixed AS STAT5A + B was not different from the growth after exposure to AS STAT5A or AS STAT5B alone.

Next, cultures in methylcellulose medium were performed using bone marrow CD34^+ cells to confirm our results obtained on MNC; high doses of AS STAT5A and scrambled were used in this case. It was noted that the clonogenic potential of myeloid and erythroid progenitors markedly decreased after the 24-h incubation with antisense against STAT5A (Fig. 6).

The analysis of the direct damage of human bone marrow MNC

The viability of the examined cells was analysed with a trypan blue exclusion test to determine the mechanism of the decreased clonogenicity of bone marrow haematopoietic cells after ODN exposure. A lower viability was noticed after AS STAT5A and AS STAT5A + B exposure in comparison to the control group but the differences observed were not statistically significant (Table 1).

Apoptosis assays

Annexin V staining Combined Annexin V and PI staining was employed to label apoptotic cells after the 24-h ODN exposure. The bone marrow MNC as well as CD34^+ cells were incubated with antisense and scrambled ODN in high doses. Sense oligonucleotides were omitted as there

Figure 6. The influence of ODNs on the clonogenicity of human bone marrow CD34⁺ cells ($n = 6$) after a 24-h incubation (myeloid – CFU-GM and erythroid – BFU-E colony formation) ($\bar{x} \pm \text{SD}$) (** $P < 0.001$): C, control cells not exposed to ODNs; AS STAT5A-H, cells exposed to antisense STAT5A ODNs (150 $\mu\text{g/ml}$); SCR-H, cells exposed to scrambled ODNs (150 $\mu\text{g/ml}$). Results are expressed as a percentage of the control values (100%).

Table 1. Viability of human BM MNC ($n = 12$) after a 24-h incubation with ODNs

	C	AS STAT5A	AS STAT5B	AS STAT5A + B	SCR
Mean ($\bar{x}$)	89.0%	80.5%	84.5%	78.0%	89.0%
SD	12.7	10.6	17.7	9.8	12.7

C = control cells not exposed to ODNs; AS STAT5A; AS STAT5B; AS STAT5A + B; SCR – cells exposed to antisense STAT5A; STAT5B or scrambled ODN sequence (150 $\mu\text{g/ml}$).

Table 2. Percentage of apoptotic cells after a 24-h incubation of human bone marrow MNC ($n = 12$) with ODNs

	C	AS STAT5A	AS STAT5B	AS STAT5A + B	SCR
Non-apoptotic cells	86.3 ± 11.1	82.8 ± 10.1	82.2 ± 22.0	77.1 ± 14.8	85.4 ± 9.8
Apoptotic cells (early stages)	7.9 ± 6.1	8.4 ± 6.4	7.3 ± 17.3	10.4 ± 11.8	6.7 ± 5.0
Apoptotic cells (late stages)	4.6 ± 3.8	5.5 ± 5.5	7.7 ± 7.9	8.0 ± 6.0	4.9 ± 4.6

C = control cells not exposed to ODNs; AS STAT5A; AS STAT5B; AS STAT5A + B; SCR = cells exposed to antisense STAT5A; STAT5B or scrambled ODN sequence (150 $\mu\text{g/ml}$) ($\bar{x} \pm \text{SD}$; Annexin V/PI staining).

had been no significant changes between control cells and cells incubated with sense ODNs in the clonogenic assays. A higher percentage of apoptotic cells was observed after incubation with antisense ODNs compared to the control in the bone marrow MNC (Table 2). However, the differences were not statistically significant. A similar result was obtained for the population of CD34⁺ cells (Table 3, Fig. 7).

Table 3. Percentage of apoptotic cells after a 24-h incubation of human bone marrow CD34⁺ (*n* = 6) cells with ODNs

	C	AS STAT5A	AS STAT5B	AS STAT5A + B	SCR
Non-apoptotic cells	72.9 ± 11.8	60.4 ± 30.1	61.1 ± 29.3	61.6 ± 19.5	73.2 ± 15.8
Apoptotic cells (early stages)	8.2 ± 3.0	16.2 ± 17.2	17.3 ± 11.4	14.3 ± 5.9	9.6 ± 4.1
Apoptotic cells (late stages)	13.3 ± 8.4	15.7 ± 13.4	17.3 ± 11.8	16.2 ± 11.9	14.2 ± 13.3

C = control cells not exposed to ODNs; AS STAT5A; AS STAT5B; AS STAT5A + B; SCR = cells exposed to antisense STAT5A; STAT5B or scrambled ODN sequence (150 µg/ml) (*x* ± SD; Annexin V/PI staining).

Figure 7. A representative study of the detection of apoptosis after 24-h incubation of human bone marrow CD34⁺ cells with ODNs (AS STAT5A, AS STAT5B, AS STAT5A + B; scrambled) (150 µg/ml) (FL1, Annexin V-FITC; FL3, PI).

Table 4. Percentage of apoptotic cells after a 24-h incubation of human bone marrow CD34⁺ (*n* = 6) cells with ODNs

	C	AS STAT5A	AS STAT5B	AS STAT5A + B	SCR
Apoptotic cells	24.1 ± 13.7	30.4 ± 14.7	32.4 ± 22.2	29.1 ± 15.3	24.2 ± 11.6

C = control cells not exposed to ODNs; AS STAT5A; AS STAT5B; AS STAT5A + B; SCR = cells exposed to antisense STAT5A; STAT5B or scrambled ODN sequence (150 µg/ml) ($\bar{x}$ ± SD; the TUNEL method).

TUNEL method A further cytometric method was applied to investigate the level of apoptosis in the population of bone marrow CD34⁺ cells. No statistically significant differences were observed in the percentage of CD34⁺ cells undergoing apoptosis between control cells not exposed to the ODNs and cells exposed to AS STAT5A and/or STAT5B and scrambled ODN for 24 h. However, apoptosis was enhanced in populations of cells that were incubated with antisense ODNs (Table 4, Fig. 8).

DISCUSSION

The role of the *STAT5* proteins in adult haematopoiesis is not clear, so we decided to explore the influence of perturbation of *STAT5* expression on the clonogenicity and apoptosis of bone marrow haematopoietic cells. Phosphorothioate antisense oligonucleotides were employed to disrupt *STAT5* gene expression at the mRNA level. The so-called 'antisense strategy' relies on specific nucleotide base pairing to target the gene of interest. Synthetic antisense oligonucleotide may be internalized by cells from the extracellular milieu and is able to hybridize within the cell to the appropriate mRNA sense sequence (Ratajczak & Gewirtz 1994; Kregenow *et al.* 1995; Gewirtz *et al.* 1998). The antisense strategy has been applied successfully to disrupt the expression of genes coding proteins such as growth factors, cellular receptors, second messengers and enzymes (Kregenow *et al.* 1995; Majka *et al.* 2000). In addition, there are many potential clinical situations where antisense ODNs may be useful therapeutically, for example in clinical oncology, viral and parasitic diseases, and in cardiovascular diseases (Ratajczak & Gewirtz 1994; Kregenow *et al.* 1995; Luger *et al.* 2002). However, studies using ODNs are limited by the effectiveness of the AS ODN, so the reverse transcription PCR method was used to show that our AS ODNs efficiently down-regulated expression of *STAT5* at the mRNA level (Luger *et al.* 2002; Majka *et al.* 2000). Moreover, we applied Western blot analysis to prove that a decrease of the *STAT5A* protein level occurred in the AS *STAT5A*-treated cells. Sense and scrambled ODNs were also employed to check any non-specific effects of these compounds (Majka *et al.* 2000).

Our experiments were performed on cells obtained from HCOD, which are a new potential source of haematopoietic stem cells (Machaliński *et al.* 2001a,b, 2003). Bone marrow haematopoietic cells can be easily aspirated from HCOD because donors are heparinized before organ donation and blood in their bone marrow cavities remains liquid. These cells have been postulated to be efficient for use in research as well as for transplantation purposes (Machaliński *et al.* 2001b).

In *ex vivo* conditions the *STAT5* proteins have been demonstrated to play a critical role in the proliferation of murine haematopoietic cells and haematopoietic cell lines after IL-3, IL-6,

Figure 8. A representative study of the analysis of apoptosis after a 24-h incubation of human bone marrow CD34⁺ cells with ODNs (AS STAT5A, AS STAT5B, AS STAT5A + B; scrambled) (150 µg/ml) (FL1-dUTP-FITC; M1, non-apoptotic cells).

GM-CSF, SCF, and FLT3 ligand stimulation (Teglund *et al.* 1998; Ilaria *et al.* 1999; Bradley *et al.* 2002). However, they did not influence cell proliferation after EPO stimulation (Teglund *et al.* 1998). In our experiments on human bone marrow MNC obtained from HCOD, perturbation of STAT5A expression decreased the clonogenicity of these cells in methylcellulose cultures

after stimulation by EPO, SCF and IL-3 or GM-CSF and IL-3, respectively. AS STAT5B failed to reduce significantly the growth of CFU-GM in contrast to BFU-E, it suggests that this protein may take part in human erythropoiesis only, but not in myelopoiesis. AS STAT5A also lowered the clonogenic potential of a pure population of early haematopoietic CD34⁺ progenitors derived from bone marrow in methylcellulose cultures. Since cultures in methylcellulose medium are thought to influence proliferation as well as differentiation of haematopoietic progenitor cells, STAT5 proteins seem to play a significant role in human haematopoiesis. We did not observe statistically significant differences between growth of cells incubated with antisense ODNs in low or high doses nor between mixed AS STAT5A + B and these antisense ODNs alone. The effect of ODNs seems to be dose-independent. Clonogenic assays were also performed on haematopoietic bone marrow cells obtained from healthy donors using the AS STAT5A ODN (data not shown), which also decreased the growth of these cells after a 24-h exposure. The viability of the examined cells did not decrease significantly after antisense ODN exposure, so the reduced proliferative potential of bone marrow cells seems not to be the result of a non-specific toxic effect of the ODNs used. On the other hand, the analysis of viability by the trypan blue exclusion test is not as sensitive a method as clonogenic assays. This may explain the non-significant influence of antisense ODNs on the viability of examined cells.

STAT5 has been shown to inhibit apoptosis in some types of cell by the influence on transcription of such genes as *bcl-2*, *bcl-x*, *pim1* (Socolovsky *et al.* 1999; Kieslinger *et al.* 2000). The process of apoptosis was enhanced in haematopoietic bone marrow cells from adult STAT5A^{-/-}B^{-/-} mice, especially in populations of more differentiated cells (Snow *et al.* 2002). On the other hand, STAT5 was reported not to play a significant role in the regulation of apoptosis of human CD34⁺ cells derived from cord blood, since the dominant negative form of STAT5 decreased *bcl-2* gene expression without affecting apoptosis (Buitenhuis *et al.* 2003). It has been reported by Kyba *et al.* that induction of STAT5 in a murine embryonic stem cell line enhanced the proliferation and differentiation of haematopoietic progenitors without altering the apoptosis of those cells (Kyba *et al.* 2003). Bone marrow MNC, as well as a population of CD34⁺ cells, were used in assays to elucidate apoptosis in our study. The population of CD34⁺ cells is thought to contain haematopoietic stem cells (Ratajczak & Gewirtz 1995), and STAT5 has been reported to be highly expressed in these cells (Buitenhuis *et al.* 2003). No statistically significant increase in the number of apoptotic cells was observed in the case of MNC or CD34⁺ cells after the antisense STAT5A and STAT5B exposure. Although we have reported that STAT5s take part in the regulation of apoptosis of selected human leukaemic cell lines (Baškiewicz-Masiuk *et al.* 2003b) and in blast cells derived from patients with acute and chronic myeloid leukaemia (manuscript submitted), they did not influence the apoptosis of normal human haematopoietic cells.

We have reported previously that antisense oligonucleotides against STAT5 mRNA did not effect human haematopoietic cells obtained from umbilical cord blood (Baškiewicz-Masiuk *et al.* 2003a). In this study, it has been shown that the antisense STAT5 ODNs inhibited the clonogenicity of bone marrow cells. In contrast to cord blood cells, HCOD-derived bone marrow cells (as the cells of an adult, older organism), have a lower proliferative potential (Traycoff *et al.* 1994; Hao *et al.* 1995). This may explain the variation between results obtained from these two studies. Moreover, bone marrow cells harvested from HCOD were isolated in a very special situation, ie close to the death of the person. Although the HCOD was still connected to a respirator and his/her blood pressure was stabilized, the central nervous system of these patients was non-functional and neuroendocrine stimulation was probably disturbed. Since the bone marrow cells are likely to have been exposed to stress condition it is possible that they might have been more sensitive to intracellular changes such as levels of signalling proteins.

In conclusion, studies using the STAT5A as well as the STAT5B antisense ODNs in human haematopoietic HCOD-derived bone marrow cells indicated that these proteins play a major role in the growth of these cells, especially those of the erythroid system, thus indicating a potential role for STAT5 proteins in human haematopoiesis, particularly in pathological conditions.

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