The influence of STAT5 antisense oligonucleotides on the proliferation and apoptosis of selected human leukaemic cell lines

M. Baśkiewicz-Masiuk*, M. Masiuk† and B. Machaliński*

*Department of General Pathology and †Department of Pathology, Pomeranian Medical University, Szczecin, Poland

Received 2 June 2003; revision accepted 19 August 2003

Abstract. The signal transducers and activators of transcription – STAT5A and STAT5B – take part in the regulation of many essential physiopathological processes. They influence the cell cycle, apoptosis and the proliferation of different types of cell lines. The STAT5 proteins are induced in response to multiple haematopoietic cytokines. Because they are constitutively active in certain haemato-oncologic diseases, it is also suggested that they play an important role in leukaemogenesis. However, function of these proteins in haematopoietic cell transformation and proliferation is not clear. The aim of this study was to evaluate the impact of perturbation of STAT5 expression [using oligodeoxynucleotide (ODN) against STAT5 mRNA], on the clonogenicity and survival of selected human leukaemic cell lines, HEL, HL-60, K562, TF-1. We analysed the effect of ODN pre-treatment on the cell clonogenicity in methylcellulose cultures according to the time and the temperature of exposure. Moreover, we attempted to estimate apoptosis induced in examined cells, by flow cytometry using combined Annexin V-PI staining and the TUNEL method. We also applied the RT-PCR method to analyse *Bax* and *Bcl-x_L* gene expression. We found that the perturbation of STAT5 expression with antisense oligonucleotides caused a decrease in the proliferative potential of human K562 and TF-1 cell lines. Also, we observed higher induction of apoptotic cell death in the K562 and TF-1 cells incubated with the antisense STAT5A ODNs. We did not notice any impact of ODNs on the HL-60 and HEL cells. Our studies using STAT5 antisense oligonucleotides showed that these proteins may be critical in the regulation of growth and apoptosis of some types of leukaemic blasts.

INTRODUCTION

Cytokines regulate many essential physiological processes including cell growth, differentiation and development (Ihle 1995; Leonard & Lin 2000; Ravandi *et al.* 2002). They are recognized by specific receptors on the cell surface and they then activate various transduction systems. One

Correspondence: Magdalena Baśkiewicz-Masiuk and Bogusław Machaliński, Department of General Pathology, Pomeranian Medical University, Al. Powstańców Wlkp.72, 70–121 Szczecin, Poland. Tel.: 004891 4661546; Fax: 004891 4661548; E-mail: poziomka@med.pam.szczecin.pl and machalin@sci.pam.szczecin.pl

of the most important signalling pathways is the JAK-STAT pathway induced by multiple extracellular polypeptides (Leonard & O'Shea 1998; Imada & Leonard 2000; Aaronson & Horvath 2002). Following binding of a ligand to its cognate receptor on the cell surface, Janus family tyrosine kinases (JAK) associated with a receptor lead to self-phosphorylation and phosphorylation of intracellular tyrosine residues on the receptor, creating STAT docking sites. STAT proteins are recruited to the receptor via the STAT SH2 domain and they are phosphorylated by JAKs. Then STATs dimerize and translocate to the nucleus where they modulate the expression of different genes through binding to target gene promoters (Imada & Leonard 2000; Aaronson & Horvath 2002). Seven STAT proteins have been identified in mammalian cells: STAT1–4, STAT5A/B and STAT6. Alterations in their activation have been demonstrated in various diseases (Durbin *et al.* 1996; Akira 1999; Bowman *et al.* 2000; Ward *et al.* 2000).

As the STAT proteins are essential for cytokine-regulated processes such as proliferation, differentiation and survival, it is suggested that they play a role in cell transformation. Many recent findings support this hypothesis (Weber-Nordt *et al.* 1998; Bowman *et al.* 2000). Enhanced STAT activation, especially of STAT3 and STAT5, may contribute to malignant transformation by promoting cell-cycle progression or cell survival (Benekli *et al.* 2003). The aberrant activation of these proteins has been observed in many malignancies, including breast cancer, head and neck cancer, and malignant melanoma as well as lymphomas and leukaemias (Bowman *et al.* 2000; Benekli *et al.* 2003). There are two STAT5 proteins, STAT5A and STAT5B, that share more than 90% identity at the amino acid level and are encoded by closely related but distinct genes (Grimley *et al.* 1999; Imada *et al.* 2000). They are activated by many haematopoietic cytokines including interleukin-3 (IL-3), granulocyte-macrophage-colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), erythropoietin (EPO), thrombopoietin (TPO), and stem cell factor (SCF). The STAT5 proteins take part in myeloid differentiation, programmed cell death and proliferation of different haematopoietic cell lines (Kieslinger *et al.* 2000; Ravandi *et al.* 2002). They seem to play a role in haematopoietic cell transformation as they are constitutively active in many haematopoietic malignancies, for example, acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML), acute lymphocytic leukaemia (ALL), human T-cell lymphoma virus type I (HTLV-1)-related adult T-cell leukaemia/lymphoma, as well as in cutaneous T-cell lymphoma (Benekli *et al.* 2003). New information concerning the function of these proteins might lead to the development of novel cancer therapies, therefore the role of STAT5 in haematopoietic cell proliferation requires further study (Seidel *et al.* 2000; Turkson & Jove 2000).

With these issues in mind, the aim of our study was to explore the influence of the STAT5A and STAT5B proteins, using the oligodeoxynucleotide (ODN) strategy against STAT5 mRNA, on proliferation and apoptosis of selected human leukaemic cell lines. We analysed clonogenicity of the HEL, HL-60, K562, TF-1 cells in methylcellulose cultures according to the type of ODN used and the time of exposure. We also attempted to estimate the level of apoptosis induced in examined cell lines after incubation with ODNs (Bařkiewicz-Masiuk *et al.* 2003).

MATERIALS AND METHODS

Cell lines

We used four different cell lines in our experiments: HEL, M6 subtype of AML in the French-American-British classification (FAB); HL-60, M3 subtype of AML in the FAB classification; TF-1, M6 subtype of AML in the FAB classification; CML cell line, K562. They were obtained

from ATCC and were maintained in RPMI-1640 supplemented with 10% foetal calf serum (FCS), 1 mg/ml L-glutamine and antibiotics. In addition, human IL-3 and GM-CSF were necessary for the growth of the TF-1 cells (Hayakawa *et al.* 1998; Liu *et al.* 1999; Gesbert & Griffin 2000). The viability of cells was assayed by a trypan blue exclusion test. The cells were counted using a haemocytometer and subsequently used for further experiments.

Oligodeoxynucleotides

Oligodeoxynucleotides (ODNs) were supplied by MWGBiotech (Ebersberg, Germany) as sterile sodium salts, endotoxin-free, lyophilized powders and were re-dissolved in Iscove's modified Dulbecco's medium (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, STAT5B and scrambled, SCR) were as previously described (Bańkiewicz-Masiuk *et al.* 2003). A scrambled ODN sequence was utilized as an additional control.

ODN exposure protocol

Briefly, $0.5\text{--}1 \times 10^6$ cells were incubated in 1 ml of phosphate-buffered saline (PBS) containing 10% FCS for 24 or 72 h at 37 °C with AS STAT5A or AS STAT5B or SCR (150 µg/ml final concentration) (Majka *et al.* 2000; Luger *et al.* 2002). After the incubation, the cells were washed twice in IMDM and subsequently their viability was assayed. Next, the cells were plated in methylcellulose cultures. The number of cells that were undergoing apoptosis was assessed by employing three separate assays. RNA was extracted from a selection of the cells for further analysis. Control groups contained cells manipulated in an identical manner but without exposure to ODNs.

STAT5 mRNA expression

After incubation with ODNs, total RNA was isolated from 1×10^6 cells 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 as described (Nosaka *et al.* 1999; Gesbert & Griffin 2000; Majka *et al.* 2000). β -actin levels were used as an internal control. Total RNA (1 µg) was reverse-transcribed to cDNA with M-MLV reverse transcriptase (Promega, Madison, WI, USA). For PCR, cDNA was amplified using 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. 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.

Cell cultures

Cells (5×10^3) were re-suspended in 0.4 ml Iscove's IMDM and mixed with 1.8 ml methylcellulose medium MethoCult HCC-4230 (StemCell Technologies Inc., Vancouver, British Columbia, Canada) supplemented with L-glutamine (0.125 mM). The appropriate growth factors were added to the mixture, which was then transferred to 3.5-cm diameter plastic Petri dishes and incubated (37 °C, 95% humidity, 5% CO₂) for the period of time appropriate for the formation of the particular colonies. Growth factors employed for colony stimulation, in the case of the cytokine-dependent TF-1 cell line, were as follows: IL-3 (20 U/ml) (Genetic Institute, R & D System, Minneapolis, MN, USA) plus GM-CSF (5 ng/ml) or EPO (5 U/ml) (Amgen Inc., Thousand Oaks, CA, USA). Recombinant human growth factors were employed in all experiments. Colonies were counted during observation under an inverted microscope on day 11. Cultures

were performed in quadruple. Results were expressed as a percentage of control values (arbitrarily taken as 100%) (Machaliński 1999).

Assays of apoptosis

Combined Annexin V-propidium iodide staining The level of apoptosis was assessed in living cultures using Annexin V (BD Biosciences, San Jose, CA, USA) following the manufacturer's specifications. Binding of fluorescein-conjugated Annexin V and propidium iodide (PI) was analysed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA) (Kieslinger *et al.* 2000; Baškiewicz-Masiuk *et al.* 2003).

The TUNEL method Apoptosis was also quantified in fixed preparations by using the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) assay (APO-DIRECT, BD Biosciences Pharmingen, USA) following the manufacturer's instructions. DNA breaks were labelled and visualized with FITC-dUTP for assessment on a FACSCalibur flow cytometer (Moulian *et al.* 2001).

Bcl-X_L and Bax gene expression Total RNA was isolated as described above. *Bcl-x_L*, *Bax* and *β-actin* levels were assessed by a semi-quantitative polymerase chain reaction as previously described (Majka *et al.* 2000). The following primers were used: *Bax* primers (forward: 5'-GGGGACGAAGTGGACAGTAA-3'; reverse: 5'-CAGTTGAAGTTGCCGTCAGA-3'), *Bcl-x_L* primers (forward: 5'-TTCAGTGACCTGACATCCCA-3'; reverse: 5'-TCCACAAAAGTATC-CCAGCC-3') (Baškiewicz-Masiuk *et al.* 2003).

Statistical analysis

Arithmetical means and standard deviations were calculated on an IBM computer using MS Excel vs. 97. The experiments were performed on each cell line with six repetitions, cells having been cultured in quadruple at each point. Data were analysed by the Mann-Whitney test. Statistical significance was defined as $P < 0.05$.

RESULTS

RT-PCR analysis of STAT5 mRNA expression in leukaemic cell lines after incubation with ODNs

It has been previously demonstrated that the anti-STAT5 ODNs employed in this study specifically inhibit their respective targets in haematopoietic cells at the mRNA level (Baškiewicz-Masiuk *et al.* 2003). Nonetheless, to confirm it, ODN-treated leukaemic cell lines were examined for STAT5 mRNA expression (Fig. 1).

The influence of ODNs on the clonogenicity of selected human leukaemic cell lines

We had performed a 24-h incubation at a temperature of 37 °C to explore the influence of the ODNs on the growth of different AML cell lines, TF-1, HL-60, HEL, and on the CML cell line, K562. The TF-1 cells, which are dependent on cytokines, were stimulated to proliferate with human IL-3 and GM-CSF after exposure to ODNs. The cytokines had also been added to the liquid medium during the time of the 24 h incubation with ODNs to investigate whether their presence would change ODN impact on the cells. Erythroleukaemic TF-1 cells can also be stimulated

Figure 1. STAT5A mRNA expression in HL-60 cells after an incubation with ODNs, gel-separated RT-PCR products. M. molecular weight DNA marker; lane 1, control cells not exposed to ODNs; lane 2, cells exposed to AS STAT5A (150 µg/ml) for 24 h; lane 3, cells exposed to SCR (150 µg/ml) for 24 h. Identical results were obtained for expression of STAT5B (data not shown).

to grow with EPO treatment, so an additional experiment on these cells was performed to explore their proliferation after incubation with AS ODNs and stimulation with EPO. It was observed that during the 24 h incubation with the antisense oligodeoxynucleotides against STAT5A, as well as STAT5B, TF-1 cells exhibited decreased clonogenicity (Fig. 2, TF-1a, -1b, -1c). Differences found between the examined groups and their controls were statistically significant in the case of the TF-1 cells incubated simultaneously with ODNs and the cytokines (STAT5A, $P < 0.05$, STAT5B, $P < 0.001$) (Fig. 2, TF-1b). Significantly lower numbers of the TF-1 colonies were also observed after incubation with STAT5A AS ($P < 0.001$) as well as STAT5B AS ($P < 0.01$) after the EPO stimulation (Fig. 2, TF-1c). Moreover, a difference was noted between proliferation of these cells after exposure to STAT5A and STAT5B AS ($P < 0.05$). The STAT5A antisense oligodeoxynucleotide was more effective at reducing proliferative potential of TF-1 cells after stimulation with EPO. Exposure to a scrambled sequence ODN had little or no effect on colony formation, suggesting sequence specificity on growth inhibition. It was observed that the HL-60 and K562 cells incubated with the antisense ODNs used had lower clonogenic growth compared with the control cells, however, the differences were not statistically significant (Fig. 2, HL-60, K562). A non-significant effect was noted in the case of the HEL cells incubated with STAT5A AS (data not shown).

As a statistically significant effect of AS ODNs was initially found only on growth of the human TF-1 cell line, duration of the experiment was extended in each case up to 72 h (Luger *et al.* 2002). Figure 3 shows the clonogenic growth of TF-1 and K562 cells after 72 h exposure

Figure 2. Influence of ODNs on the clonogenicity of human leukaemic cell lines after 24 h incubation ($\pm$ SD) ($n = 6$): TF-1a, cultures in methylcellulose stimulated to proliferate with IL-3 and GM-CSF; TF-1b, cultures stimulated with IL-3 and GM-CSF together during the incubation period and in methylcellulose; TF-1c, cells stimulated with EPO; HL-60 clones, K562 clones.

Figure 3. Influence of ODNs on the clonogenicity of human leukaemic cell lines: TF-1 (IL-3, GM-CSF treated) and K562 after 72-h incubation ($\pm$ SD) ($n = 6$).

to ODNs. A significant decrease of proliferation in the TF-1 cells (STAT5A, $P < 0.005$; STAT5B, $P < 0.01$) and the K562 cells (STAT5A, $P < 0.01$; STAT5B, $P < 0.001$) treated with the AS ODNs was observed. Extending the time of incubation also influenced HL-60 cells, and a decrease in clonogenicity was significant after incubation with STAT5A ($P < 0.05$), STAT5B

Table 1. Percentage of apoptotic cells after 24-h incubation of human leukaemic cell line TF-1 with ODNs

	C	STAT5A	STAT5B	SCR
Non-apoptotic cells	75.7% $\pm$ 13.0%	54.1% $\pm$ 21.8%	60.0% $\pm$ 19.6%	70.9% $\pm$ 14.7%
Apoptotic cells (early stage)	8.3% $\pm$ 6.8%	15.4% $\pm$ 10.9%	19.2% $\pm$ 13.2%	8.8% $\pm$ 9.9%
Apoptotic cells (late stage)	11.7% $\pm$ 10.6%	23.7% $\pm$ 8.2%	15.7% $\pm$ 10.8%	10.0% $\pm$ 10.9%

C, control cells not exposed to ODNs, AS STAT5A; AS STAT5B; SCR, cells exposed to antisense STAT5A; STAT5B or scrambled ODN sequence, respectively (150 μ g/ml) ($\pm$ SD) (Annexin V-PI staining).

($P < 0.005$) and SCR ($P < 0.05$). This effect did not seem to be specific for the STAT5 antisense oligonucleotides. A statistically significant effect was not detected on the HEL cells after prolonged incubation with AS ODNs, however, a lower number of colonies was observed in the case of exposure to AS STAT5A (data not shown).

Assays of apoptosis

Combined Annexin V-propidium iodide staining Apoptosis was analysed by Annexin V and PI staining using flow cytometry. This method is useful to distinguish early stages from late stages of the process. The highest and statistically significant incidence of apoptosis was noticed in the TF-1 cells after AS STAT5A exposure, but not after AS STAT5B exposure ($P < 0.05$) (Table 1, Fig. 4). Slight, but not significant, differences were observed in percentages of cells undergoing apoptosis between the control cells and the HL-60 and K562 cells exposed for 24 h to AS STAT5A and STAT5B. The differences were clearly evident in the late stages of apoptotic cell death.

The TUNEL method A further cytometric method has been used to investigate apoptosis amongst the four leukaemic cell lines. The percentage of apoptotic cells was higher ($P < 0.01$) in the population of the TF-1 cells after incubation with STAT5A AS (Table 2, Fig. 5). AS STAT5A also increased the number of apoptotic cells in the population of K562 cells, however, this was not found to be statistically significant. No differences were detected in percentages of HL-60 and HEL cells undergoing apoptosis after 24 h exposure to AS STAT5A, STAT5B or SCR ODNs.

Bcl-X_L and Bax gene expression Expression levels of the pro-apoptotic *Bax* and anti-apoptotic *Bcl-x_L* genes in the subject cells were investigated after 24 h incubation with ODNs using the RT-PCR method. No differences in expression of the mRNAs from these genes was observed between controls and HL-60 and HEL cells exposed to ODNs. Expression of *Bax* gene increased in the TF-1 cells, however, after the incubation with antisense ODNs, in particular, STAT5A AS (data not shown). Figure 6 shows the expression of β -actin (control), *Bax* and *Bcl-x_L* mRNAs in the population of K562 cells. RT-PCR performed on these showed enhanced expression of the *Bax* gene after incubation with AS STAT5A and also decreased expression of anti-apoptotic *Bcl-x_L*.

DISCUSSION

STAT proteins, through binding to promoters of different genes, can increase the proliferative potential and inhibit apoptosis of different types of cells. STATs, especially STAT3 and STAT5,

Figure 4. A representative study for the detection of apoptosis after 24-h incubation of human TF-1 cells with ODNs (AS STAT5A; AS STAT5B; SCR) (FL1, Annexin V FITC; FL3, PI).

Table 2. Percentage of apoptotic cells after a 24-h incubation of leukaemic TF-1 cell line with ODNs

	C	STAT5A	STAT5B	SCR
Apoptotic cells	1.4% ± 1.0%	9.2% ± 3.8%	2.7% ± 2.4%	1.6% ± 0.9%

C, control cells not exposed to ODNs, AS STAT5A; AS STAT5B; SCR, cells exposed to antisense STAT5A; STAT5B or scrambled ODN sequence (150 µg/ml) (± SD) (TUNEL method).

have also been found to be associated with viral or oncogene-mediated transformation (Weber-Nordt *et al.* 1998; De Groot *et al.* 1999; Bowman *et al.* 2000; Schwaller *et al.* 2000). Moreover, blocking constitutively active STAT using dominant-negative STAT mutants in transformed cells has caused cell-cycle arrest and apoptosis (Gesbert *et al.* 2000; Kieslinger *et al.* 2000).

Constitutive activation of STAT5 has been demonstrated in some leukaemic cell lines, as well as in peripheral blood cells derived from patients with AML and CML (Gouilleux-Gruart

Figure 5. A representative study for the detection of apoptosis after 24-h incubation of human TF-1 cells with ODNs (AS STAT5A; AS STAT5B; SCR) (FL1, dUTP FITC).

et al. 1996; Shuai *et al.* 1996; Xia *et al.* 2001a,b; Spiekermann *et al.* 2002). To date, it remains unknown which of these two proteins, STAT5A or STAT5B, is responsible for the changes in haematopoietic cells, which may cause transformation. In mouse, transplants of cells expressing a constitutively active mutant of STAT5A caused development of foetal myeloproliferative disease (Schwallier *et al.* 2000). However, *in vivo* studies using STAT5B were not performed in that study. The dominant negative form of STAT5A has been shown to inhibit Ba/F3 cell proliferation mediated by TEL-JAK2 (Lacronique *et al.* 2000). In contrast, STAT5B takes part in the control of proliferation of cells of human head and neck squamous cell carcinoma (Leong *et al.* 2002). The *STAT5B* gene has also been newly identified fused to the *RAR α* gene in acute promyelocytic leukaemia (APL) (Arnould *et al.* 1999). The function of the STAT5 protein, however, requires further study.

Antisense oligodeoxynucleotides can be effective molecular tools to investigate the function of proteins in cells (Turkson & Jove 2000). The influence of STAT5A and STAT5B suppression has been estimated, using the oligodeoxynucleotide strategy, on proliferation of selected AML cell lines, HL-60, HEL, TF-1, and the CML cell line, K562. Cultures in methylcellulose were used after the exposure to ODNs for (i) 24 h and (ii) 72 h. Apoptosis of these cells was investigated

Figure 6. Gel-separated RT-PCR products. β -actin (control of mRNA integrity); *Bax*; and *Bcl-x_L* mRNA expression in human K562 cells after 24-h incubation with ODNs. M, molecular weight DNA marker; 1, β -actin: A, control cells not exposed to ODNs; B, cells exposed to AS *STAT5A* ODNs (150 μ g/ml); C, cells exposed to AS *STAT5B* (150 μ g/ml); D, cells exposed to SCR (150 μ g/ml); 2, *Bax*: A, control cells not exposed to ODNs; B, cells exposed to AS *STAT5A* ODNs (150 μ g/ml); C, cells exposed to AS *STAT5B* (150 μ g/ml); D, cells exposed to SCR (150 μ g/ml); 3, *Bcl-x_L*: A, control cells not exposed to ODNs; B, cells exposed to AS *STAT5A* ODNs (150 μ g/ml); C, cells exposed to AS *STAT5B* (150 μ g/ml); D, cells exposed to SCR (150 μ g/ml); H_2O .

by three methods: (i) Annexin V staining, (ii) the TUNEL method and (iii) analysis of *Bax* and *Bcl-x_L* gene expression by RT-PCR after 24 h of ODN pre-treatment. Apoptosis is characterized by a loss of membrane phospholipid asymmetry. Phosphatidylserine (PS), which is located in an inner leaflet of the plasma membrane, translocates to the surface of the cell, thereby playing an important role in removal of apoptotic bodies by the surrounding cells (Israels & Israels 1999). As Annexin V has an affinity to PS, staining the cells with fluorescein isothiocyanate-labelled Annexin V enables the detection of apoptotic cells by flow cytometry (Koopman *et al.* 1994). Changes in structure of the cell membrane are considered to be characteristic of early stages of apoptosis. Annexin V results were confirmed by the TUNEL assay. This method detects DNA fragmentation through a reaction catalysed by TdT, which incorporates dUTP into the 3' – OH ends of double- and single-stranded DNA. In a mechanistic means of identifying apoptosis, RT-PCR estimation of *Bax* and *Bcl-x_L* gene expression was used. These have been found to be regulated by haematopoietic growth factors. Mice nullizygous for both *STAT5A* and *STAT5B* genes have shown decreased expression of the *Bcl-x_L* protein (Santos *et al.* 2001).

The role of *STAT5* in AML cell lines is not clear. TF-1, HL-60 and HEL lines were employed to identify the effect of *STAT5* gene suppression on different populations of AML cells. The TF-1 cell line alone was haematopoietic growth factor-dependent, thus, to investigate, cell proliferation cultures were stimulated with IL-3 and GM-CSF, or with EPO. A significant decrease of TF-1 cell proliferation was observed, which was more evident after 72-h exposure. IL-3 and GM-CSF were also applied during incubation with ODNs. Antisense ODNs caused a more intensified reduction in cells growth compared with proliferation of cells incubated with ODNs without cytokines. This result could be explained as stimulation such as the cytokines activating *STAT5* proteins, so more evident blocking effect of AS ODNs (Nosaka *et al.* 1999). EPO was used to stimulate the TF-1 cells to proliferate after 24 h exposure to ODNs. *STAT5* proteins have been shown to be required for the survival of EPO-dependent red blood cell precursors (Socolovsky *et al.* 1999; Bromberg & Darnell 2000). In the TF-1, erythroleukaemia cell line,

EPO sustains short-term growth and causes differentiation along the erythroid lineage (Chretien *et al.* 1996). However, this cell line possesses a truncated EPO receptor, and stimulation by EPO induced an impaired STAT5 activation in these cells (Chretien *et al.* 1996). In our experiment, a negative effect of AS STAT5A and AS STAT5B was observed on the growth of the TF-1 cells, which was significantly greater in the case of the STAT5A antisense ODN. Using the Annexin V assay, numbers of apoptotic TF-1 cells were higher after exposure to AS ODNs. However, the differences were statistically significant only after AS STAT5A exposure. This increase in numbers of apoptotic cells after incubation with AS STAT5A was also detected by the TUNEL assay. Enhanced pro-apoptotic *Bax* gene expression was also observed after AS ODN exposure. No changes in the expression of *Bcl-x_L* were found, but regulation of this gene expression also requires other than STAT5 signalling pathways (Gesbert *et al.* 2000). Studies using IL-3-dependent cell lines showed that the dominant negative (DN) form of STAT5 inhibited growth of these cells, suggesting that STAT5 is needed for their proliferation (Ilaria *et al.* 1999; Coffey *et al.* 2000). DN STAT5A mutants have also been reported to suppress IL-3-induced proliferation without inducing apoptosis (Sillaber *et al.* 2000). An earlier finding concerning a dominant negative form of STAT5 protein showed that DN STAT5 is able to reduce, but not suppress, factor-dependent growth of haematopoietic cell lines (Kieslinger *et al.* 2000). In our studies, the negative effect of antisense ODNs, especially AS STAT5A, was noted on this cytokine-dependent cell line compared with the HEL and HL-60 lines. This result suggested that these signalling factors are important for the proliferation and survival of the TF-1, growth factor-dependent cells.

A non-statistically significant influence of AS ODNs on HL-60 – promyelocytic, and HEL – erythroleukaemic cell lines was observed after 24-h exposure. Prolonged pre-treatment caused a decrease in the numbers of HL-60 colonies. However, this effect was noticed in the cells incubated with STAT5A AS, STAT5B AS and SCR, which suggested a non-specific effect that may depend on the impact of chemical modification of the compounds used. However, the HL-60 cell line is the M3 subtype of AML in the FAB classification and these cells have a constitutively active form of STAT5, so the block effect might even so be specific (Coffey *et al.* 2000). No changes in the growth of the HEL cells were found, other than a minor effect on proliferation after 72 h exposure to AS STAT5A. HEL, like HL-60, possesses a constitutively active form of STAT5, however, both are *Bcr/Abl* negative (Liu *et al.* 1999; Nosaka *et al.* 1999). The Annexin V assay suggested a higher rate of apoptosis of the HL-60 cells exposed to AS ODNs, but this was not statistically significant. No effect was observed on HEL apoptotic cell death using this method. The TUNEL assay did not reveal an influence of AS ODNs on apoptosis of the HEL or HL-60 cells. No difference was visible in the expression of pro- and anti-apoptotic genes in the individual groups of examined HEL, HL-60 cells. All our findings concerning these two AML cell lines showed that the STAT5 proteins did not play any significant role in HL-60 and HEL cell growth and survival. Genetic and functional abnormalities, which result in constitutive activation of STAT5 are not known in these two cell lines, but constitutive activation of the transcription factors is triggered by a mechanism other than the *Bcr/Abl* oncogene. Constitutive phosphorylation of the Raf-1 protein occurs in HEL, but not in Meg-01 cells, which are *Bcr/Abl* positive (Liu *et al.* 1999). Thus it seems that Raf-1, but not STAT5, may participate in the HEL cell proliferation.

K562 is a human Philadelphia (Ph) chromosome-positive CML cell line. The Ph chromosome is formed after translocation between the chromosomes 9 and 22, t(9 : 22), takes place, and generates the *Bcr/Abl* gene. This is associated with development of chronic myeloid leukaemia (CML) (Gesbert *et al.* 2000). The protein product of this gene – p210^{*Bcr/Abl*} – displays enhanced tyrosine kinase activity that constitutively phosphorylates STAT5 proteins (Shuai *et al.* 1996; De Groot *et al.* 1999). *Bcr/Abl* presence increases cell proliferation and prolongs viability

of transformed cells (Sillaber *et al.* 2000). Moreover, it converts IL-3-dependent cell lines to growth factor independence. *Bcr/Abl* is also thought to reduce apoptotic cell death through modulation of anti-apoptotic *Bcl-x_L* gene expression, of which the promoter contains STAT5 binding sites (Gesbert *et al.* 2000; Kieslinger *et al.* 2000). The dominant negative STAT5 isoform has been reported to inhibit proliferation of *Bcr/Abl* transformed cells. In contrast to these findings, *Bcr-Abl*-mediated transformation has been stated to occur in cells deficient for STAT5A/5B (Sexl *et al.* 2000). It has been shown that *Bcr/Abl* activates STAT5 weakly in contrast with other leukaemic protein tyrosine kinases (TEL-JAK2, TEL-ABL) (Spiekermann *et al.* 2002). In our studies, a minor decrease in K562 cell proliferation was observed after 24 h incubation with antisense ODNs. The influence of AS ODNs was higher, being statistically significant (STAT5A, $P < 0.01$; STAT5B, $P < 0.001$) after 72 h incubation. *Bcr/Abl* activates multiple other signalling proteins besides STAT5 transcription factors, including Ras, PI3-kinase, c-Jun and c-Myc (Weber-Nordt *et al.* 1998; Gesbert *et al.* 2000). The constitutive form of STAT5 protein alone is not as potent as the *Bcr/Abl* product in proliferation support, and the inhibition of any single pathway in *Bcr/Abl* transformed cells is unlikely to eliminate the viability effect of this oncogene (Shuai *et al.* 1996; Sillaber *et al.* 2000). Three dominant negative forms of proteins taking part in the *Bcr/Abl*-mediated leukaemogenesis are DN Ras, DN STAT5 and DN phosphatidylinositol 3-kinase, which inhibit the growth of K562 by 90, 55 and 40%, respectively (Sonoyama *et al.* 2002). Activation of further signalling proteins might be an explanation for lack of statistically significant blocking of the K562 growth after 24 h incubation with AS STAT5.

By Annexin V staining, no significantly higher number of apoptotic cells was noted in the group of K562 cells exposed to AS ODNs. This was confirmed after AS STAT5 pre-treatment in the TUNEL assay. However, the RT-PCR method, which is thought to be more sensitive, indicated alterations in anti- and pro-apoptotic gene expression. Apoptosis was assessed after 24-h ODN exposure here. It is conceivable that measurement of apoptosis after 72 h incubation with ODNs might show greater changes, however, this study investigated early stages of the process only.

Studies using ODNs are limited by the effectiveness of the AS ODNs, but here we have shown, using the RT-PCR method, that our antisense oligonucleotides decreased expression of the *STAT5* gene (Majka *et al.* 2000; Luger *et al.* 2002). We also employed scrambled oligonucleotides to check the non-specific effect of these compounds (Majka *et al.* 2000).

In conclusion, our studies using the STAT5A and STAT5B antisense oligodeoxynucleotides in selected human haematopoietic cell lines indicated that they play a role in regulation of proliferation and apoptotic cell death in selected AML and CML human leukaemic cell lines. However, other signalling proteins are also necessary for their growth. Cellular transformation is a complex process involving multiple signalling pathways, and the inhibition of one of them may be inefficient in totally reducing viability and proliferative potential of the cells. Constitutive activation of STAT5 may be triggered by different mechanisms in various cell lines and cell-type specificity may play a key role in STAT5 signalling.

REFERENCES

- AARONSON DS, HORVATH CM (2002) A road map for those who don't know JAK-STAT. *Science* **296**, 1653.
- AKIRA S (1999) Functional roles of STAT family proteins: lessons from knockout mice. *Stem Cells* **17**, 138.
- ARNOULD C, PHILIPPE C, BOURDON V, GREGOIRE MJ, BERGER R, JONVEAUX P (1999) The signal transducer and activator of transcription *STAT5b* gene is a new partner of retinoic acid receptor alpha in acute promyelocytic-like leukaemia. *Hum. Mol. Genet.* **8**, 1741.

- BAŚKIEWICZ-MASIUK M, MASIUK M, DOMAGAŁA W, MACHALIŃSKI B (2002) The role of STAT5 proteins in the regulation of normal hematopoiesis and selected hematoneoplastic diseases. *25th Meeting of the European Study Group for Cell Proliferation, Abstract no. 09*, France: Reims.
- BAŚKIEWICZ-MASIUK M, MASIUK M, CZAJKA R, MACHALIŃSKI B (2003) The role of STAT5 proteins in the regulation of normal hematopoiesis in a cord blood model. *Cell Mol. Biol. Lett.* **8**, 317.
- BENEKLI M, BAER MR, BAUMANN H, WETZLER M (2003) Signal transducer and activator of transcription proteins in leukemias. *Blood* **101**, 2940.
- BOWMAN T, GARCIA R, TURKSON J, JOVE R (2000) STATs in oncogenesis. *Oncogene* **19**, 2474.
- BROMBERG J, DARNELL JE JR (2000) The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* **19**, 2468.
- CHRETIEN S, VARLET P, VERDIER F, GOSBERT S, CARTRON JP, GISSELBRUCHT S, MAYEUX P, LACOMBE C (1996) Erythropoietin-induced erythroid differentiation of the human erythroleukemia cell line TF-1 correlates with impaired STAT5 activation. *EMBO J.* **15**, 4174.
- COFFER PJ, KOENDERMAN L, DE GROOT RP (2000) The role of STATs in myeloid differentiation and leukemia. *Oncogene* **19**, 2511.
- DE GROOT RP, RAAIJMAKERS J, LAMMERS J, JOVE R, KOENDERMAN L (1999) STAT5 activation by BCR-Abl contributes to transformation of K562 leukemia cells. *Blood* **94**, 1108.
- DURBIN JE, HACKENMILLER R, SIMON MC, LEVY DE (1996) Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* **84**, 443.
- GESBERT F, GRIFFIN JD (2000) Bcr/Abl activates transcription of the *Bcl-X* gene through STAT5. *Blood* **96**, 2269.
- GOUILLEUX-GRUART V, GOUILLEUX F, DESAINT C, CLAISSE JF, CAPIOD JC, DELOBEL J, WEBER-NORDT R, DUSANTER-FOURT I, DREYFUS F, GRONER B, PRIN L (1996) STAT-related transcription factors are constitutively activated in peripheral blood cells from acute leukemia patients. *Blood* **87**, 1692.
- GRIMLEY PM, DONG F, RUI H (1999) Stat5a and Stat5b: fraternal twins of signal transduction and transcriptional activation. *Cytokine Growth Factor Rev.* **10**, 131.
- HAYAKAWA F, TOWATARI M, IIDA H, WAKAO H, KIYOI H, NAOE T, HIDEHIKO S (1998) Differential constitutive activation between STAT-related and neutrophil proteins and MAP kinase in primary acute myelogenous leukemia. *Br. J. Haematol.* **101**, 521.
- IHLE JN (1995) Cytokine receptor signaling. *Nature* **377**, 591.
- ILARIA RL, HAWLEY RG, VAN ETEN RA (1999) Dominant negative mutants implicate STAT5 in myeloid cell proliferation and neutrophil differentiation. *Blood* **93**, 4154.
- IMADA K, LEONARD WJ (2000) The Jak-Stat pathway. *Mol. Immunol.* **37**, 1.
- ISRAELS L, ISRAELS E (1999) Apoptosis. *Stem Cells* **17**, 306.
- KIESLINGER M, WOLDMAN I, MORIGGL R, HOFMANN J, MARINE JC, IHLE JN, BEUG H, DECKER T (2000) Antiapoptotic activity of STAT5 required during terminal stages of myeloid differentiation. *Genes Dev.* **14**, 232.
- KOOPMAN G, REUTELINGSPERGER CP, KUIJTEN GA, KEEHNEN RM, PALS ST, VAN OERS MH (1994) Annexin V for flow cytometric detection of phosphatidyl-serine expression on B cells undergoing apoptosis. *Blood* **84**, 1415.
- LACRONIQUE V, BOUREUX A, MONNI R, DUMON S, MAUCHAUFFE M, MAYEUX P, GOUILLEUX F, BERGER R, GISSELBRECHT S, GHYSDAEL J, BERNARD OA (2000) Transforming properties of chimeric TEL-JAK proteins in Ba/F3 cells. *Blood* **95**, 2076.
- LEONARD WJ, LIN JX (2000) Cytokine receptor signaling pathways. *J. Allergy Clin. Immunol.* **105**, 877.
- LEONARD WJ, O'SHEA JJ (1998) Jaks and STATs: biological implications. *Annu. Rev. Immunol.* **16**, 293.
- LEONG PL, XI S, DRENNING SD, DYER KF, WENTZEL AL, LERNAR EC, SMITHGALL TE, GRANDIS JR (2002) Differential function of STAT5 isoforms in head and neck cancer growth control. *Oncogene* **21**, 2846.
- LIU RY, FAN C, GARCIA R, JOVE R, ZUCKERMAN KS (1999) Constitutive activation of the JAK2/STAT5 signal transduction pathway correlates with growth factor independence of megakaryocytic leukemic cell lines. *Blood* **93**, 2369.
- LUGER SM, O'BRIEN SG, RATAJCZAK J, RATAJCZAK MZ, MICK R, STADTMAUER EA, NOWELL PC, GOLDMAN JM, GEWIRTZ AM (2002) Oligodeoxynucleotide-mediated inhibition of *c-myc* gene expression in autografted bone marrow: a pilot study. *Blood* **99**, 1150.
- MACHALIŃSKI B, SZOŁOMICKA P, KJOWSKI J, BAŚKIEWICZ M, KARBICKA A, BYRA E, MAJKA M, GIEDRYS-KALEMBA S, RATAJCZAK MZ (1999) Short-term storage of human haematopoietic cells. Influence of air and deoxyribonuclease I. *Ann. Transplant.* **4**, 29.
- MAJKA M, RATAJCZAK J, MACHALIŃSKI B, CARTER A, PIZZINI D, WLSIK MA, GEWIRTZ AM, RATAJCZAK MZ (2000) Expression, regulation and function of AC133, a putative human haematopoietic cells. *Folia Histochem. Cytobiol.* **38**, 53.
- MOULIAN N, TRUFFAULT F, GAUDRY-TALARMAIN YM, SERRAF A, BERRIH-AKNIN S (2001) *In vivo* and *in vitro* apoptosis of human thymocytes are associated with nitrotyrosine formation. *Blood* **97**, 3521.

- NOSAKA T, KAWASHIMA T, MISAWA K, IKUTA K, MUI A, KITAMURA T (1999) STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells. *EMBO J.* **18**, 4754.
- RAVANDI F, TALPAZ M, KANTARJIAN H, ESTROV Z (2002) Cellular signaling pathways: new targets in leukaemia therapy. *Br. J. Haematol.* **116**, 57.
- SANTOS SC, LACRONIQUE V, BOUCHAERT I, MONNI R, BERNARD O, GISSELBRECHT S, GOUILLEUX F (2001) Constitutively active STAT5 variants induce growth and survival of hematopoietic cells through a PI 3-kinase/Akt dependent pathway. *Oncogene* **20**, 2080.
- SCHWALLER J, PARGANAS E, WANG D, CAIN D, ASTER JC, WILIAMS IR, LEE CH, GERTHNER R, KITAMURA T, FRANTSVE J, ANASTASIADOU E, LOH ML, LEVY DE, IHLE JN, GILLIAND G (2000) Stat5 is essential for the myelo- and lymphoproliferative disease induced by TEL/JAK2. *Mol. Cell.* **6**, 693.
- SEIDEL HM, LAMB P, ROSEN J (2000) Pharmaceutical intervention in the JAK/STAT signaling pathway. *Oncogene* **19**, 2645.
- SEXL V, PIEKORZ R, MORIGGL R, ROHRER J, BROWN MP, BUNTING KD, ROTHAMMER K, ROUSSEL MF, IHLE JN (2000) Stat5a/b contribute to interleukin 7-induced B-cell precursor expansion, but *abl*- and *bcr/abl*-induced transformation are independent of Stat5. *Blood* **96**, 2277.
- SHUAI K, HALPERN J, HOEVE J, RAO X, SAWYERS CL (1996) Constitutive activation of STAT5 by the *BCR-ABL* oncogene in chronic myelogenous leukemia. *Oncogene* **13**, 247.
- SILLABER C, GESBERT F, FRANK DA, SATTTLER M, GRIFFIN JD (2000) STAT5 activation contributes to growth and viability in Bcr/Abl-transformed cells. *Blood* **95**, 2118.
- SOCOLOVSKY M, FALLON AE, WANG S, BRUGNARA C, LODISH HF (1999) Fetal anemia and apoptosis of red cell progenitors in Stat5a/-5b/- mice: a direct role for Stat5 in Bcl-X_L induction. *Cell* **98**, 181–191.
- SONOYAMA J, MATSUMURA I, EZOE S, SATOH Y, ZHANG X, KATAOKA Y, TAKAI E, MIZUKI M, MACHII T, WAKAO H, KANAKURA Y (2002) Functional cooperation among Ras, STAT5, and phosphatidylinositol 3-kinase is required for full oncogenic activities of BCR/ABL in K562 cells. *J. Biol. Chem.* **277**, 8076.
- SPIEKERMANN K, PAU M, SCHWAB R, SCHMIEJA K, FRANZRAHE S, HIDDEMANN W (2002) Constitutive activation of STAT3 and STAT5 is induced by leukemic fusion proteins with protein tyrosine kinase activity and is sufficient for transformation of hematopoietic precursor cells. *Exp. Hematol.* **30**, 262.
- TURKSON J, JOVE R (2000) STAT proteins: novel molecular targets for cancer drug discovery. *Oncogene* **19**, 6613.
- WARD A, TOUW I, YOSHIMURA A (2000) The Jak-Stat pathway in normal and perturbed hematopoiesis. *Blood* **95**, 19.
- WEBER-NORDT RM, MERTELSMANN R, FINKE J (1998) The JAK-STAT pathway: signal transduction involved in proliferation, differentiation and transformation. *Leuk. Lymphoma* **28**, 459.
- XIA Z, SALZLER RR, KUNZ DP, BAER MR, KAZIM L, BAUMANN H, WETZLER M (2001a) A novel serine-dependent proteolytic activity is responsible for truncated signal transducer and activator of transcription proteins in acute myeloid leukemia blasts. *Cancer Res.* **61**, 1747.
- XIA Z, SAIT SN, BAER MR, BARCOS M, DONOHUE KA, LAWRENCE D, FORD LA, BLOCK AMW, BAUMANN H, WETZLER M (2001b) Truncated STAT proteins are prevalent at relapse of acute myeloid leukemia. *Leuk Res.* **25**, 473.