

Plasma CD105, TGF β -1, TGF β -3 and the Ligand/Receptor Complexes in Children with Acute Lymphoblastic Leukaemia

ABDULFATTAH AL-MOWALLAD¹, TREVOR CARR², ABDULLAH AL-QOUZI¹,
CHENGGANG LI¹, RICHARD BYERS^{1,3} and SHANT KUMAR^{1,4}

¹Division of Laboratory and Regenerative Medicine, The University of Manchester, Oxford Road, Manchester, M13 9PT;

²Department of Clinical Haematology, Royal Manchester Children Hospital, Hospital Road, Manchester, M27 1HA;

³Department of Pathology, Manchester Royal Infirmary Hospital, Oxford Road, Manchester, M13 9WL;

⁴Christie Hospital, Wilmslow Road, Manchester, M20 4BX, U.K.

Abstract. *The role of angiogenesis in solid tumours is well recognised, but its importance in haematological malignancies is less well understood. In leukaemia, mainly the determination of microvascular density utilising immunohistochemistry in the bone marrow trephines and the measurement of soluble angiogenic factors have led to the recognition that angiogenesis may be important in leukaemia as well. In this study, the soluble form of the endothelial cell activation/proliferation (i.e., angiogenesis) marker CD105 and its ligands TGF β -1 and -3, as well as the ligand/receptor complexes in plasma from children with acute lymphoblastic leukaemia (ALL) were quantified. The plasma level of CD105 was significantly higher in patients with common ALL compared to controls, while the TGF β -3 level was lower in patients. Neither the CD105 or TGF β -3 levels were of prognostic value, nor did they correlate with any of the known prognostic indicators, such as white blood cell counts. There were no significant differences between the plasma levels of any of the other parameters, such as TGF β -1 or the ligand receptor complexes, in children with leukaemia compared to controls. Our results support the role of angiogenesis in leukaemia and suggest that anti-angiogenesis may be a therapeutic target in leukaemia.*

Solid tumours are dependent on angiogenesis for their growth and metastasis [reviewed in (1)]. Evidence has also begun to emerge that angiogenesis may be important in haematological malignancies (2). These studies in leukaemia have utilised mainly 2 approaches viz immunohistochemistry to quantify

Correspondence to: Shant Kumar, Department of Pathology, Medical School, Manchester University, Manchester M13 9PT, U.K. Tel: 00 44 161275-5298, Fax: 00 44 161275-5289, e-mail: shant.kumar@manchester.ac.uk

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microvessel density (MVD) in bone marrow biopsies using pan endothelial cell markers and the measurement of soluble pro-angiogenic factors in the blood and urine of patients with leukaemia.

Pule *et al.* (3), utilising anti-factor VIII-related antigen and anti-thrombomodulin antibodies demonstrated increased MVD in the pre-treatment bone marrow biopsies from children with acute lymphoblastic leukaemia (ALL), compared to the normal aged matched control group which returned to normal in remission. In this study no survival advantage for MVD was found. These authors also observed that the difference in MVD between patients and controls was due to the presence of small aberrantly immature blood vessels in leukaemic marrows. Another publication, using computer-aided 3-dimensional reconstruction of bone marrow vasculature showed that microvessels in the bone marrow of children with ALL resembled those in solid tumours (4). Similarly, in bone marrow biopsies from patients with acute myeloid leukaemia (AML), higher MVD in patients than controls which also returned to within the normal range after complete remission were reported. On day 16 post-chemotherapy, Padro *et al.* (5) noted that MVD was reduced by 60% and 17% in the hypoplastic marrows of patients without and with residual disease, respectively. This increase in MVD was due to the production of angiogenic factors by the leukaemic cells (*e.g.* vascular endothelial growth factor mRNA and protein) (6). Likewise, bone marrow biopsies from patients with chronic lymphoblastic leukaemia (CLL) (7), chronic myeloid leukaemia (CML) and myelodysplastic syndrome showed evidence of increase in angiogenesis by immunohistochemistry (8).

However, the quantification of angiogenesis utilising immunohistochemistry in the bone marrow has major drawbacks. First, the size of the biopsy is usually relatively very small compared to the bone. Hence there is a high probability that the test biopsy may not be representative of the true picture of the bone vascular network. Second,

markers used to date to determine MVD are pan endothelial markers, which mainly stain normal microvessels and less efficiently stain angiogenic blood vessels within bone marrow. These pan-endothelial markers may also be expressed by some haematopoietic stem cells yielding false high counts, especially when computer-aided image analysis is used. Finally inter-personal discrepancies can occur when vessel count is made by different investigators (2, 9).

The second approach to studying angiogenesis in patients is by measuring the angiogenic factors in the body fluids, such as sera and urine. With regard to the source of angiogenic factors in blood, Northern blots from cultured leukaemic blasts from patients AML contained significantly higher amounts of VEGF compared to controls. In addition to VEGF, some AML blasts expressed its receptors (VEGFR-1 and VEGFR-2). In co-cultures of AML blasts and endothelial cells, the production of VEGF by the blast cells induced the secretion of granulocyte-macrophage colony-stimulating factor, indicating a paracrine mode of action for VEGF in AML (10). The plasma levels of VEGF and bFGF (another potent angiogenic factor) were significantly higher in patients with AML and MDS compared to controls. The higher levels of VEGF were associated with shorter relapse-free-intervals and lower complete remission rates in patients with AML but not in MDS (11). Using immunohistochemistry, the expressions of VEGF and VEGFR-2 were significantly elevated in the bone marrow of patients with AML and their expressions correlated with bone marrow MVD (12). Real time quantitative PCR for cellular VEGF in newly diagnosed ALL and children with recurrent ALL showed a higher level in the latter compared to the former. In this study, children with higher than the median VEGF values had a shorter relapse-free interval and overall survival (13). Surprisingly, the plasma VEGF levels in children with ALL were lower than in controls but during remission were similar to those of the normal controls (14). Furthermore, although bFGF was higher in patients compared to controls, those with the low bFGF had a poor outcome. Could it be that leukaemic cells grow independently of the blood levels of angiogenic factors (15) or is their level masked by the formation of receptor/ligand complexes as we have found in patients with breast cancer (16)?

An ideal marker of angiogenesis would be one which actually reflects the angiogenic activity in the bone marrow of patients with leukaemia, either in their body fluids and/or their tissues. One promising potential candidate is the endothelial cell (EC) activation/proliferation marker CD105 (endoglin), which is a receptor for transforming growth factor β (TGF β)-1 and-3 and is involved in cell proliferation, attachment and survival of EC (reviewed in (17)). Soluble CD105 (sCD105) is detectable in conditioned media of EC and in plasma of patients with cancer and other cardiovascular diseases (18). CD105 is highly expressed on

angiogenic endothelial cells and is an independent prognostic factor in several malignancies of solid tissues (9, 17, 19).

Here, the plasma levels of CD105, TGF β -1, TGF β -3 and the receptor/ligand complexes *viz* CD105/TGF β -1 and CD105/TGF β -3 were quantified in children with ALL and the data were evaluated for their prognostic significance.

Materials and Methods

Plasma samples. At the time of diagnosis, the blood samples in EDTA containers were collected from 60 children with different subtypes of ALL (45 from patients with common ALL (cALL) and 15 with other types of ALL) and the plasma was separated and stored at -70°C until tested.

Plasma CD105, TGF β -1, TGF β -3 and the ligand/receptor complexes were determined using in-house ELISAs.

ELISA for CD105. A 96-well clear microtiter plate (Flow Laboratories Inc. Mclean, VA, USA) was coated with 100 μl /well of E9 antibody diluted in 0.1 PBS and incubated overnight at 4°C in a humidified chamber. The coated plate was blocked with 100 μl /well of 0.1% BSA(w/v) in 0.1 M PBS and 0.1% Tween 20 (Sigma-Aldrich Ltd, St. Louis, MO, USA) for 3 hours at room temperature. A standard curve was generated by serially diluting recombinant human CD105 (R&D Systems Minneapolis, USA) starting from 100 ng/ml. Test samples diluted in PBS/Tween were added to the coated plate in duplicate and incubated overnight in a humidified chamber at 4°C . After overnight incubation, 100 μl /well of biotinylated E9 antibody diluted in PBS/Tween were added to the plate, followed by 100 μl /well of horseradish peroxidase-streptavidin (R&D Systems). Three washes with PBS/Tween were carried between each of the procedures. For colour development, 100 μl /well of the SureBlue Elite substrate (Insight Biotechnology Ltd, Middlesex, UK) were added and the plate was incubated at room temperature for 20 min. The reaction was stopped by adding 1N H_2SO_4 . The absorbance was measured in an ELISA reader at 450 nm wave-length. The test sample's concentration was determined against a standard curve produced using recombinant CD105.

ELISA for TGF β -1. The total TGF β -1 was determined after releasing the bound TGF β -1 from its carrier protein by incubating the patient's plasma with 1M HCl for 20 min with shaking at room temperature and the acid was then neutralized with an equal volume of 1 M NaOH.

The ELISA procedure to measure active and acid activated TGF β -1 in the plasma was carried as follows: a 96-well clear plate was coated with 100 μl /well of mouse monoclonal anti-TG β -1 antibody (R&D Systems) and incubated overnight in a humidified chamber at 4°C . The plate was then blocked with 100 μl /well of 0.1% BSA (w/v) in 0.1 M PBS and 0.1 Tween 20 at room temperature for 3 hours. To generate a standard curve, recombinant human mature TGF β -1 (R&D Systems) was serially diluted in PBS/Tween starting from 1000 pg/ml. The activated samples were added in twice diluted 1/2 in PBS/Tween and the plate was incubated overnight in a humidified chamber at 4°C . Biotinylated chicken IgY anti-TG β -1 (R&D Systems) (100 μl /well) was added and the plate was incubated at room temperature for 3 hours. Three washes with PBS/Tween were carried between each of the steps. HRP-streptavidin (R&D Systems) was added and the plate was incubated for 30 min with shaking at room temperature. After washing, 100 μl /well of the

Table I. A summary of the plasma levels of CD105, TGFβ-1, TGFβ-3 and the ligand/receptor complexes in children with leukaemia.

Children with		CD105 (ng/ml)	TGFβ-1 (pg/ml)	TGFβ-3 (pg/ml)	CD105/TGFβ-1 (Unit)	CD105/TGFβ-3 (Unit)
ALL (n=60) (Total)	Mean±STD	48.5±114	364±490	156±424	43.6±85.3	46.7±97.1
	Median (Range)	14 (0-630)	200 (0-1900)	0 (0-1900)	5 (0-200)	0 (0-320)
	<i>p</i> value compared to controls	0.16	0.34	0.003	0.21	0.92
cALL (n=45)	Mean±STD	57±130	303±457	183±486	50±96.2	47.3±101
	Median (Range)	14 (0-630)	120 (0-1800)	0 (0-1900)	14 (0-300)	0 (0-320)
	<i>p</i> value compared to controls	0.048	0.9	0.006	0.29	0.96
Controls	Mean±STD	17.1±17.9	238±305	224±302	14.2±19.5	14.4±12.4
	Median (Range)	11 (0-60)	98 (0-1253)	125 (0-1500)	0 (0-100)	0 (0-100)
	Correlation with survival <i>p</i> value (Cox regression)	0.76	0.7	0.6	0.43	0.44

SureBlue Elite substrate (Insight Biotechnology Ltd, Wembley, Middlesex, UK) was added and the plate was incubated for 20 min at room temperature in the dark. The reaction was stopped by adding 100 µl/well of 1N H₂SO₄ and the absorbance was immediately measured at 460 nm wave-length. The absorbance of the recombinant TGFβ-1 was plotted against its concentration and the concentrations of the test samples were determined from this curve.

ELISA for TGFβ-3. TGFβ-3 was released from its carrier protein by incubating the plasma with H₂PO₄ buffer pH 2 for 20 min with shaking at room temperature. The acid was then neutralised with equal volume of H₂PO₄ buffer pH 12. The ELISA procedure for TGFβ-3 was the same as that for TGFβ-1, except that the coating antibody was mouse anti TGFβ-3 (R&D Systems) and the detection antibody was biotinylated goat anti-TGFβ-3 (R&D Systems).

ELISA for CD105/TGFβ-1 and CD105/TGFβ-3 complexes. The ELISA for determining the levels of CD105/TGFβ-1 and CD105/TGFβ-3 complexes in native plasma were undertaken without activation. The procedure was similar to the ELISA for TGFβ-1 and TGFβ-3 except that the detection antibody was replaced by biotinylated Mab E9.

Statistical analysis. All data were analysed using non-parametric statistical tests. For survival data, the Cox regression analysis was used and for correlation analysis the Spearman's test was used. Any *p* value ≤0.05 was considered significant.

Results

The results of CD105, TGFβ-1, TGFβ-3 and the ligand receptor complexes in patients with ALL are presented in

Table I. There were no significant differences between patients and controls in the plasma levels of CD105, TGFβ-1, CD/TGFβ-3 or the CD105/TGFβ-3 complexes. While the level of TGFβ-3 was significantly lower in patients with ALL compared to controls, the median and range for patients and controls were 0 (0-1900) and 0 (0-1500), respectively (*p*=0.003 Mann Whitney *U*-test).

In a major subgroup of 45 children with cALL, the CD105 levels were significantly elevated compared to controls (*p*=0.048) and the TGFβ-3 levels were significantly lower in patients compared to controls (*p*=0.006). There were no significant differences in the plasma levels for any of the other parameters between patients and controls.

In uni-variant and multi-variant analysis, apart from WBC counts, none of the other parameters was a significant predictive factor of survival.

Discussion

An increase of angiogenesis in the bone marrows of patients with different types of leukaemia is strongly suggestive of its role in their pathogenesis. In this study, sCD105, a marker of endothelial cell activation/proliferation was significantly elevated in the plasma of children with cALL. These results support the finding by Pule *et al.* (3), who demonstrated an increase in MVD in children with cALL. The observed difference between patients and normal controls was mainly due to the small immature vessels, *i.e.*, vessels undergoing angiogenesis. Previously, it was observed that CD105 plasma

levels significantly decreased in the post-chemotherapy samples from patients with different types of solid tumours (20). In this study, paired samples were not available to compare CD105 levels in pre- and post-chemotherapy specimens and therefore it is not possible to say whether levels of sCD105 would return to normal values in patients at remission. We found that sCD105 was not a prognostic indicator of survival. Pule and colleagues (3) also had noted that MVD did not correlate with survival. In our study there was no significant correlation between sCD105 plasma levels and WBC counts (3). Similarly, angiogenesis did not correlate with the cellularity of the bone marrow in patients with AML but did with the percent of blasts in the bone marrow (6).

The fact that there is increased angiogenesis in the bone marrow of patients with leukaemia, points to likelihood of the potential usefulness of anti-angiogenic therapy in haematological malignancies. Thalidomide, an angiogenesis inhibitor, gave encouraging results in the treatment of myeloma and some other angiogenesis inhibitors have also shown promise in earlier in phase I clinical trials (2).

CD105 is a receptor for TGF β -1 and -3 and its expression by endothelial cells antagonises their inhibitory effect on EC (17, 21). TGF β -1 inhibits WBC proliferation, induces maturation of immature cells and inhibits the proliferation of some leukaemia cells, such as B-CLL (22), myeloid (23) and lymphoid leukaemia (24). In our patients, the mean and median values of the TGF β -1 and CD105/TGF β -1 complex were higher in the patients than in the controls, however this difference was not statistically significant probably due to the relatively small number of patients examined. Malignant cells can escape the inhibitory effect of TGF β -1 either by down-regulating the expression of one of its receptors or TGF β signalling proteins (25). In CLL, although TGF β -1 inhibits the proliferation of some leukaemic cell lines, one-third of blasts isolated from patients with adult CLL were resistant to TGF β -1-induced inhibition as they had lost TGF β -R1 (26). Our results suggest an early loss of the TGF β receptor, hence insensitivity of the ALL blasts to its inhibitory effect. Or these high levels of TGF β -1 in children with ALL, compared to the low levels in adults, indicates a difference in the biology of leukaemic disease in adult and children (26). In our patients, the expression of TGF β -R1 in leukaemic cells was not examined and we speculate that the loss of the TGF β receptor occurs in patients with ALL; those with high TGF β -1 may have lost the receptor. The source of the TGF β -1 may be the leukaemic blasts themselves, as in the B cells in CLL (26), or the bone marrow stroma (27). The high level of TGF β -1 in these patients may offer a growth advantage to the resistant leukaemic cells over normal cells by inhibiting lymphokine-activated natural killer cells, as in myeloblastic leukaemia

(28). TGF β -1 is angiogenic *in vivo* (29), hence high TGF β -1 levels may induce more angiogenesis in the bone marrow of patients with ALL.

The mean value for plasma CD105/TGF β -3 was higher in the patients compared to the controls, while the median value was the same. The higher levels of the complexes in patients is probably due to higher values of CD105, which significantly correlated with levels of the 2 complexes ($p=0.001$, for both types of complexes). There was also a strong correlation between the TGF β -3 levels and those of the CD105/TGF β -3 complex ($p\leq 0.001$, $r=0.5$ Spearman's test). The exact function of these soluble complexes is a matter of speculation; it is possible that the formation of these complexes may reduce the amount of active TGF β -1 and -3 and hence antagonise their inhibitory effect on cells.

This is the first report that has examined TGF β -3 levels in leukaemia. Interestingly, the TGF β -3 plasma levels were significantly lower in patients compared to controls ($p=0.006$). TGF β -3 is produced by tumour stroma (30) and is associated with lymphatic metastasis and a shorter survival time in patients with breast cancer (31). *In vitro* TGF β -3 inhibited the proliferation of CML blasts (32). The effect of TGF β -3 was three- to five-fold more potent than TGF β -1 in osteoblasts due to its higher affinity for all 3 types of TGF β -receptors (TGF β -RI, TGF β -RII and CD105) (33). Whether TGF β -3 production was suppressed by leukaemic cells, was a result of the replacement of the bone marrow stroma by the tumour cells or was due to gene(s) disruption which predisposes patients to the development of leukaemia will need further investigation. The increase in TGF β -1 and the decrease in TGF β -3 in children with ALL as well as the fact that both cytokines bind to the same receptors indicates different regulatory roles for the 2 isoforms in these patients.

In conclusion, we have demonstrated that CD105 levels were higher and that TGF β -3 levels lower in children with cALL compared to controls, but neither had any prognostic significance. The quantification of ligand-receptor complexes showed a trend for higher levels of TGF β -1 and the 2 complexes in patients compared to controls, but the difference was not statistically significant.

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