TGFα expression in myeloid malignancies

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ABSTRACT

Background Transforming growth factor α (TGFα) is a member of the epidermal growth factor (EGF) family of peptide growth factors. Binding of TGFα to its cell surface ligand, EGF receptor (EGFR), results in receptor dimerisation and tyrosine kinase activity. This leads to activation of downstream signalling pathways, including Ras/Raf/MAPK and PI3K/Akt, resulting in cell proliferation and differentiation. TGFα is present in embryogenesis and is transiently expressed in some foetal tissues where it controls epidermal development. It is a recognised growth factor in chicken erythropoiesis, maintaining progenitor self-renewal, with withdrawal resulting in terminal erythroid differentiation. TGFα is also reported to be expressed by promyelocytes, myelocytes, eosinophil precursors and megakaryocytes. It is not expressed by mature neutrophils or lymphoid cells in the BM, lymph node or spleen. TGFα expression is also found in multiple non-haemopoietic tissues, including epithelial cells, smooth muscle and brain, where it plays a physiological role in angiogenesis, wound healing and neural cell proliferation. TGFα plays a role in bone remodelling by inhibiting chondrogenesis and stimulating bone resorption through effects on osteoclast development.

In addition to its physiologic role, TGFα may play a role in a number of pathological processes. Mutations in the TGFA gene, which encodes TGFα, have been implicated in some cases of nonsyndromic orofacial clefts. In mouse models TGFα signalling contributes to progressive pulmonary fibrosis through EGFR activation. TGFα is expressed in several malignancies (eg, gastric carcinoma and melanoma) where TGFα–EGFR signalling may drive tumour growth via autocrine growth factor mechanisms.

Despite the known expression of TGFα in normal myelopoiesis and that it is implicated in non-haemopoietic malignancy, there are no studies assessing TGFα expression in myeloid malignancies. In this study, we have assessed, using an immunohistochemical approach, the expression of TGFα in BM biopsies from patients with acute and chronic myeloid malignancies. Detection of TGFα in haemopoietic malignancies could have both diagnostic and therapeutic implications.

METHODS

Samples BM trephine biopsy specimens from patients with acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML), myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN) and non-malignant controls were selected for study. Table 1 lists the diagnostic subclassification and number of cases within each of these categories. The study had ethical approval and all patients had given consent for their BM to be studied.

Immunohistochemical staining BM specimens were sectioned at 4 μm and placed on positively charged slides for immunohistochemical analysis on a BOND RX automated immunostainer (Leica Biosystems, Australia). Normal kidney was included in all staining experiments. Positive (renal tubule) and negative (interstitial cell) staining was clearly differentiated. Omission of primary antibody on a renal section was used as a negative control to exclude non-specific antibody binding. All slides were dewaxed in Bond dewax solution (product code AR9222, Leica Biosystems) before undergoing heat-induced epitope retrieval at pH 9 for 20 min (Bond epitope retrieval solution 2, product code AR9640, Leica Biosystems) during the automated staining process. TGFα antibody (clone 213-4.4, product code MA1-91686, Thermo Scientific, USA) was diluted 1:300 in Bond primary antibody diluent (product code AR9332, Leica Biosystems) and incubated on BM sections for 30 min. An alkaline phosphatase Bond Polymer Refine Red Detection kit (product code D9390, Leica Biosystems) was used to detect antigen binding. Sections were counterstained in haematoxylin and mounted in Ultramount 4 mounting media (Hurst Scientific, Australia).
### RESULTS

**Non-malignant disorders**

Good quality staining with clear distinction of positive and negatively stained cells was observed with the TGFα antibody in positive control tissue; negative controls showed no staining. In normal and reactive BMs TGFα staining was present in the erythroid lineage throughout differentiation, megakaryocytes, early granulopoiesis, osteoblasts and osteoclasts, in keeping with published literature (figure 1). In the erythroid lineage staining intensity increased to a maximum at the late normoblast stage. Anucleate erythrocytes were positive though less intense than the late normoblasts. Scattered blast cells in normal marrow biopsies were weakly positive but staining decreased throughout granulopoiesis; promyelocytes, myelocytes and mature granulocytes were negative. Osteoblasts and osteoclasts, present in small numbers in the normal and reactive marrows, were TGFα positive. We confirmed this reactivity in the cases of renal osteodystrophy and Paget’s disease of bone. Osteoclasts were of moderate staining intensity whereas osteoblasts were intensely positive. All identified blood vessels demonstrated moderate intensity TGFα immunoreactivity in smooth muscle cells. Skeletal muscle, where present, demonstrated moderate intensity TGFα staining.

**Myeloid malignancies**

*Acute myeloid leukaemia*: leukaemic myeloblasts were TGFα positive in 28/30 (93%) cases of AML. The staining was weak in 10/28 (36%) and of moderate intensity in 18/28 (64%) cases. All blast cells were uniformly positive within a specimen (figure 2). Three of the 32 AML cases were erythroleukaemia (two erythroid/myeloid and one pure erythroid leukaemia); all demonstrated moderate intensity staining of the leukaemic blasts and strong staining of dispersed, maturing and erythroid precursors. Of the 30 AML cases, there were nine cases of APML. These showed overall weaker TGFα staining than other AML cases (figure 2) and two were negative. The mean staining intensity of the blasts in APML was 1.0 compared with 1.8 in the other AML cases (p=0.0004; figure 3).

*Chronic myeloid leukaemia*: twelve cases of CML-chronic phase (CML-CP), all of which had <5% morphologically identifiable blast cells, were evaluated. The blast cells were weakly positive (10/12; 83%) or negative (2/12; 17%); maturing granulocytic cells were uniformly negative. In keeping with normal marrow, the megakaryocytes were weakly positive (figure 4). The blast cells, all previously demonstrated to be of myeloid origin, in CML-accelerated phase (CML-AP) and CML-blast phase (CML-BP) were universally TGFα positive. The mean blast staining intensity was 0.8 in CML-CP compared with 1.7 in CML-AP (p<0.0001) and 1.8 in CML-BP (p<0.0001; figure 3).

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**Table 1** List of cases studied

| Category                  | Subtype                                   | Number |
|---------------------------|-------------------------------------------|--------|
| Non-malignant             | Normal/reactive bone marrow               | 55     |
|                           | Paget’s disease of bone                    | 7      |
|                           | Renal osteodystrophy                      | 7      |
| AML                       | AML, not otherwise specified              | 21     |
|                           | Acute promyelocytic leukaemia             | 9      |
| Chronic myeloid leukaemia | Chronic phase                             | 12     |
|                           | Accelerated phase                         | 12     |
|                           | Blast phase                               | 21     |
| Myelodysplastic syndromes | Various WHO subtypes                      | 13     |
| Myeloproliferative neoplasms | Polycythemia vera                      | 7      |
|                           | Essential thrombocythaemia                | 32     |
|                           | Myelofibrosis                             | 26     |
|                           | Systemic mastocytosis                     | 4      |
| Total                     |                                           | 226    |

AML, acute myeloid leukaemia.

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**Cytochemistry**

Tartrate-resistant acid phosphatase (TRAP) staining was performed to identify osteoclasts in some BM sections. Dewaxed BM sections were incubated in 0.01% Naphthol AS-MX phosphate substrate in ethylene glycol monoethyl ether in buffer (100 mM acetate buffer, 50 mM sodium tartrate dihydrate, 0.22% glacial acetic acid and pH 5.0) for 1 h at 37°C, counter-stained in haematoxylin and mounted in Ultramount Aqueous Permanent Mounting Medium (DAKO, Australia) for light microscopy.

**Microscopy**

All stained sections were assessed by light microscopy using an Olympus BX53 microscope (Olympus Imaging Australia, Australia) and photographed using a Pixera Pro 600ES microscope camera (Pixera, USA). TGFα positivity in myeloid blasts was scored on a 0–3 point system where no staining scored 0, weak staining scored 1, moderate staining scored 2 and strong staining scored 3.

**Statistics**

Student’s two-tailed t test was used to evaluate differences in mean staining intensity between blasts in the different phases of CML and between AML/acute promyelocytic leukaemia (APML) cases.

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**Figure 1** Normal bone marrow stained for transforming growth factor α. (A) Erythroid cells are moderately to strongly positive (white arrow) and megakaryocytes are weakly positive (black arrow). Magnification ×600. (B) Intensely positive osteoclast adjacent to trabecular bone (white arrow) with moderate intensity staining in multiple osteoblasts (black arrow). Magnification ×400.
Myelodysplastic syndromes: erythroid cell staining was comparable in intensity to normal BM but the staining highlighted abnormally large or dispersed erythroid islands. Dysplastic megakaryocytes, confirmed by morphology and CD61 positivity, were weakly to moderately positive in 12/13 (92%) specimens. In one BM (refractory cytopenia with multilineage dysplasia) there were intensely TGFα positive interstitially located large cells with monolobated nuclei. These were confirmed to be dysplastic megakaryocytes by CD61 positivity and TRAP negativity. All cases with an identifiable blast component demonstrated weak staining of these cells; mature granulocytes were negative.

Myeloproliferative neoplasms: since TGFα was positive in normal megakaryopoiesis, we studied a large number of MPN cases (n=65) with megakaryocytic hyperplasia to determine if there was any alteration in expression. No differences were seen: the megakaryocytes showed the same weak positivity for TGFα as seen in normal and reactive marrows, including immune thrombocytopenia. There was no association between staining intensity and MPN subtype, megakaryocyte size, location or morphology. In systemic mastocytosis (n=4) the neoplastic spindle-shaped mast cells were weakly TGFα immunoreactive. Osteoblasts and osteoclasts were prominent with staining intensity in keeping with normal marrow.

DISCUSSION
In this study, we have demonstrated that TGFα is expressed in early myeloid progenitors as evidenced by expression in normal and neoplastic myeloblasts. TGFα is retained through erythroid and megakaryocytic differentiation. In erythropoiesis the intensity of TGFα increases from early to late normoblastic differentiation whereas expression is unaltered throughout megakaryopoiesis. TGFα is expressed early in granulocytic differentiation and is lost following differentiation from the myeloblast to promyelocyte. Of greatest significance is that TGFα is expressed by neoplastic myeloid blasts. This was seen in all myeloid malignancies with a blast cell component, that is, AML, CML-AP, CML-BP and MDS. Significant differences were seen between APML and other types of AML. We propose that this reflects the greater degree of granulocytic differentiation in the malignant promyelocytes of this form of leukaemia. There were also significant differences in intensity of TGFα expression between the accelerated and blast phases of CML compared with the blast cells in chronic phase. Also of note is the fact that the cases of MDS in which there were an identifiable blast cell component demonstrated weak TGFα positivity, similar to that seen in normal BM specimens. Cases of AML generally demonstrated moderate-intensity TGFα positivity.

The precise cause for these findings is unclear from this study. While it is possible that TGFα plays a role in disease acceleration (eg, through previously suggested growth factor activity), other factors such as progressively diminished differentiation capability (and cytoplasmic accumulation of TGFα) and
differential activation of intracellular signalling pathways may all contribute to heightened TGFα staining in more acute leukaemia. Further work is required to clarify the precise mechanism/s underlying this change.

Since TGFα is highly expressed in myeloblasts, it may be a useful immunohistochemical blast cell ‘biomarker’ in BM specimens. In contrast to CD34, which is known to only be expressed in approximately 50% of AML cases, TGFα was positive in 91% of AML cases we studied and showed uniform positivity throughout the leukemic population. This makes it a robust marker with greater sensitivity than CD34 in BM specimens. Highlighting this was the finding that six cases of AML in our cohort were negative for both CD34 and CD117; TGFα was positive in all six (100%) of these cases. This demonstrates the potential of TGFα as a useful addition to the immunohistochemical repertoire for analysis of myeloid malignancies.

TGFα immunostaining could also be used to identify a potential immunotherapeutic target. Although specific TGFα inhibitors are not presently available for clinical use, several specific EGFR inhibitors are available. These agents have shown activity in cases of AML,12,13 as well as preventing or reversing pulmonary fibrosis in animal models.14 This signalling axis may, therefore, be involved in the development of BM fibrosis.

In summary, TGFα is expressed in normal and neoplastic myeloblasts. These data have potential clinical application in both diagnostic capacity (as a blast cell ‘biomarker’) and as a potential new therapeutic approach. Further study is required to determine whether targeted therapy may play a role in targeted treatment of the myeloid disorders.

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Take home messages

- TGFα is expressed in multiple myeloid lineages; intensity of expression varies by lineage and degree of maturation.
- TGFα expression is significantly stronger in AML than APML and accelerated/blastic phase CML than chronic phase CML.
- TGFα immunohistochemistry may be a useful addition to diagnostic haematopathology.

Handling editor Mary Frances McMullin
Contributors SK undertook morphological review and drafted the manuscript. BM performed all immunohistochemical staining and reviewed the manuscript. KF obtained ethics approval for study and reviewed the manuscript. WNE undertook morphological review and reviewed the manuscript.

Competing interests None declared.

Ethics approval Sir Charles Gairdner Human Research Ethics Committee.

Provenance and peer review Not commissioned; externally peer reviewed.

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