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bjh research paper

Regulation of angiogenesis in the bone marrow of myelodysplastic syndromes transforming to overt leukaemia

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Summary

To investigate the regulatory mechanisms of angiogenesis in the development of myelodysplastic syndromes (MDS) and its progression to overt leukaemia (OL), bone marrow samples from control, paired samples from MDS patients before and after transformation to OL (MDS → OL) and *de novo* acute myeloid leukaemia (AML) were analysed. Immunohistochemical staining showed a significant increase of bone marrow microvascular density (MVD) in MDS and *de novo* AML compared with controls. Surprisingly, in MDS, MVD significantly decreased upon transformation to OL, which was also significantly lower than the MVD of *de novo* AML. This evidence was strengthened by the pattern of angiogenic mediator gene expression, confirming the importance of various angiogenic mediators including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), tumour necrosis factor α (TNFα), hepatocyte growth factor (HGF) and the angiopoietin family of mediators (Ang-1 and Ang-2) as well as the receptors for angiogenic mediators, such as VEGF receptor 2 (VEGFR2) and the tyrosine kinase receptor, TIE2. By contrast, the anti-angiogenic mediator, transforming growth factor-β (TGFβ) exhibited significantly higher expression in the bone marrow of MDS → OL, indicating the importance of this cytokine as the suppressive factor of angiogenesis in MDS. These findings indicate that the bone marrow microenvironment in MDS → OL and *de novo* AML differs remarkably, suggesting the different efficacy of anti-angiogenic therapy between *de novo* AML and leukaemia secondary to MDS.

Keywords: myelodysplastic syndromes, overt leukaemia, bone marrow, angiogenesis, microvasculature.

The myelodysplastic syndromes (MDS) are a heterogeneous group of clonal haemopoietic disorders, characterised by peripheral blood cytopenia of one or more lineages and, paradoxically in the majority of cases, a normocellular or hypercellular bone marrow, this finding being mainly caused by ineffective haemopoiesis (Mufti, 2004). The pathogenesis of MDS is a multi-step process, whereby a series of DNA mutations occur within a haemopoietic stem cell that adversely affect its differentiation and maturation, leading to aberrant cellular function including susceptibility to apoptosis. Tumour suppressor inactivation, telomere shortening and silencing of DNA mismatch repair genes increase genomic instability and cytogenetic abnormalities, contributing to disease progression and leukaemic transformation (Albitar *et al*, 2002; Mufti, 2004; Disperati *et al*, 2006).

Angiogenesis is the formation of new blood vessels from pre-existing vessels (Aguayo *et al*, 2000) and is understood to be involved in the growth, dissemination and metastasis of solid tumours (Folkman, 1971). A tumour up to 2 mm in size can absorb enough oxygen and nutrients by simple diffusion from the surrounding vasculature; however, a greater vascular network is required as the tumour grows (Moehler *et al*, 2003). Haematological malignancies do not develop in the same way as solid tumours so the requirement of angiogenesis for growth and development has not been as readily recognised as for other malignancies (Bertolini *et al*, 2000; Moehler *et al*, 2003).

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However, Aguayo *et al* (2000) evaluated the importance of angiogenesis in leukaemia and MDS, and discovered increased vascularity, indicating that angiogenesis might have a role in the pathogenesis of haematological malignancies, initiating interest in this field.

Angiogenic growth factors include vascular endothelial growth factor (VEGF) (Orpana & Salven, 2002; Distler *et al*, 2003; Podar & Anderson, 2005), basic fibroblast growth factor (bFGF) (Moehler *et al*, 2003), tumour necrosis factor alpha (TNFα) (Stifter *et al*, 2005), tumour growth factor beta (TGFβ) (Distler *et al*, 2003), hepatocyte growth factor (HGF; thought to upregulate VEGF expression) (Van Belle *et al*, 1998; Bouis *et al*, 2006) and angiopoietin-1 (Ang-1) (Distler *et al*, 2003; Yu, 2005). VEGF and bFGF are the strongest inducers of angiogenesis (Albitar, 2001; Aguayo *et al*, 2003) and are synthesised in various types of cells (Moehler *et al*, 2003). The angiopoietins have both pro- and anti-angiogenic effects, acting via the tyrosine kinase receptor (TIE2). Ang-1, a pro-angiogenic factor causes autophosphorylation of TIE2 leading to endothelial cell sprouting and vessel stabilisation. Angiopoietin-2 (Ang-2) can act in opposite ways: it antagonises Ang-1, disrupting TIE2 activation and causing vessel destabilisation and regression; conversely, in the presence of VEGF, it facilitates endothelial cell proliferation (i.e. pro-angiogenic) (Distler *et al*, 2003; Tait & Jones, 2004). When there is an imbalance, angiogenesis is up- or downregulated in the mechanism described as the 'Angiogenic switch' (Lim & Levine, 2005). It is proposed that a larger vascular network provides an increased supply of oxygen and nutrients, facilitating the growth of the haematological clone and cells, such as osteoclasts, that produce pro-angiogenic factors, thus increasing angiogenesis further in a positive feedback circuit.

There is conflicting evidence regarding angiogenesis in MDS; some studies have proposed that bone marrow microvascular density (MVD) increases with MDS progression (Moehler *et al*, 2003; Wimazal *et al*, 2006), whereas others suggest increased vascularity in the early but not the latter stages of MDS (Campioni *et al*, 2004; Lundberg *et al*, 2006). Greater understanding of the role that angiogenesis plays in MDS will enable the development of therapies that can be targeted to the angiogenic pathway, thereby slowing disease progression and offering new hope to those patients for whom there are currently limited therapeutic options (Estey, 2004). The present investigation aimed to evaluate the changes in microvasculature and expression of angiogenic mediators during the development of MDS, and thus assess the role that angiogenesis plays in the progression of this disease.

Material and methods

Patients

Formalin-fixed paraffin-embedded bone marrow aspiration samples from 10 individuals with no morphological abnormalities were included as controls [mainly, patients with suspected idiopathic thrombocytopenic purpura (ITP) but with no morphological abnormality, male:female, 10 : 0; age, median 63 years (range 51–76 years)], 10 patients with MDS [three with refractory anaemia (RA), seven with RA with excess blasts (RAEB); male:female, 7:3; age, median 68 years (range, 48–77 years)] who developed overt leukaemia [OL, also classified as French–American–British (FAB) M2] later in the disease course (duration, 3 months to 3 years), and eight patients with *de novo* acute myeloid leukaemia (AML) [M2 according to the FAB classification, male:female, 4:4; age, median 60 years (range, 49–76 years)] were analysed. Age-matched control cases were included in order to exclude the influence of ageing on bone marrow cells. Diagnoses were based on standard clinical and laboratory criteria, including cell morphology (Bennett *et al*, 1982; Harris *et al*, 1999). All bone marrow aspirates were taken from the sternum and performed at the Japanese Red Cross Medical Centre, Tokyo, Japan. Control, MDS and *de novo* AML samples were collected at the time of the initial aspiration biopsy. The *de novo* AML samples exhibited a proliferation of blast cells accounting for >80% of all bone marrow cells. The patients were not infected with specific viruses, including human T cell leukaemia virus type 1 (HTLV-1), and had not been treated with therapeutic drugs prior to the study. After the initial diagnosis, MDS patients had received non-specific treatments, such as transfusion in case of severe anaemia and antibiotics or antipyretics when infectious symptoms appeared. However, they had not received any anti-cancer drugs even at the time of sample collection for OL. Bone marrow samples from MDS cases were also taken and analysed when the patients developed OL (MDS → OL). In addition, fresh frozen bone marrow samples from controls [nine cases, male:female, 4:5; age, median 62 (range, 44–87 years)], 10 paired MDS and MDS → OL patients and eight *de novo* AML cases described above were used for polymerase chain reaction (PCR) experiments.

The procedures were followed in accordance with the ethical standards established by the ethics committee of Tokyo Medical and Dental University.

Immunohistochemistry for measuring bone marrow MVD

Formalin-fixed tissue sections (10 μm) of bone marrow from controls (10 cases), MDS (10 cases) and *de novo* AML cases (eight cases) were prepared on slides covered with adhesive. Sections were deparaffinised, and endogenous peroxidase was quenched with 1·5% hydrogen peroxide in methanol for 10 min. Antibodies were applied to identify microvasculature. Primary antibodies included polyclonal rabbit antibody against von Willebrand factor (human vascular-associated antigens Factor VIII; Dako Cytomation, Glostrup, Denmark) and polyclonal rabbit antibody against VEGF receptor 2 (VEGFR2; Cell Signalling Technology, Tokyo, Japan). All sections were developed using biotin-conjugated secondary antibodies against rabbit IgG followed by a sensitive peroxidase-conjugated streptavidin system (Dako) with 3,3′ diaminobenzidine (DAB) as the chromogen. Negative control staining was

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performed using rabbit immunoglobulin of irrelevant specificity substituted for the primary antibody.

The MVD was measured using methods described previously (Padro *et al*, 2000), systematically scanning the section to find the area of most intense vascularisation (hot spot), then counting the number of vessels and measuring vessel area at 20 × power objective lens, using the Leica Quantimet 600 high resolution image analysis system (Leica, Cambridge, UK). All the patients in the study were sampled at the time of initial diagnosis and at the time of OL development. One slide at a time was observed and the areas were measured in more than five fields per slide. The MVD was calculated using the equation: Relative vascular area (%) = (total area of blood vessels)/(total area examined) × 100.

Preparation of RNA and quantitative assay for angiogenic mediators using TaqMan reverse transcription polymerase chain reaction (RT-PCR)

RNA was extracted from frozen bone marrow samples of control subjects with no morphological abnormality, MDS patients with transformation to OL, and *de novo* AML patients using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's directions. For quantitative RT-PCR, fluorescent hybridisation probes and the *Taq*Man PCR Core Reagents Kit with Ampli*Taq* Gold (PerkinElmer Cetus, Norwalk, CT, USA) were used with the ABI Prism 7900HT Sequence Detection System (PerkinElmer). Oligonucleotides (as specific primers) and *Taq*Man probes for the angiogenic mediators and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesised at a commercial laboratory (PerkinElmer Cetus). The primers and *Taq*Man probes were as follows. The sequence of the forward primer for VEGF mRNA was 5′-GACC GCGACTCGGAGAGATG-3′ and that of the reverse primer, 5′-ATGTCCCTACTCCTGTGGCC-3′; the *Taq*Man probe was 5′-GGTCCAGTCTGCCTGTCTTTCTGTCTAGT-3′. For bFGF mRNA, the forward primer was 5′-GCCGTACATCGAGTACT AGATAC-3′ and the reverse primer, 5′-TCACTCGTTTAGAC GGGACG-3′; the *Taq*Man probe was 5′-ACAAAGATACA GCACCTTCGT-3′. For TNFα mRNA, the forward primer was 5′-GCTCAGACCCGTCCAGATGAAA-3′ and the reverse primer, 5′-AGGTTGGAAGGGTTTGCG-3′; the *Taq*Man probe was 5′-AGTAACGGGACACTCCTCCTGCTTGT-3′. The forward primer for HGF mRNA was 5′-CTCCGGTACCACGA-TATGAGAAC-3′ and the reverse primer, 5′-CGTTAATTTTGT ACGCGACTGT-3′; the *Taq*Man probe was 5′-GGGAGTGT GGGCGACCCTCATGACT-3′. The forward primer for TGFβ mRNA was 5′-GAGAGGCTGGACGGTGTCT-3′ and the reverse primer, 5′-TCTAGCGCGGGTAGATCCAA-3′; the *Taq*Man probe was 5′-GGGATAAGTTCTGGTGGGTGGAAGA CCA-3′. The forward primer for Ang-1 mRNA was 5′-TCA CATAGGGTGCAGCAATCAG-3′ and the reverse primer, 5′-GT AGGCACATTGCCCATGTTG-3′; the *Taq*Man probe was 5′-CCGAAGTCCAGAAAACAGTGGGAGAAGATATA AC-3′. The forward primer for Ang-2 mRNA was 5′TTCCT CCTGCCAGAGATGGA-3′ and the reverse primer, 5′-TGCA-CAGCATTGGACACGTA-3′; the *Taq*Man probe was 5′-AA-CTGCCGCTCTTCCTCCAGCCC-3′. The forward primer for TIE2 mRNA was 5′-ACTTCGGTGCTACTTAACAACTTA-CATC-3′ and the reverse primer, 5′-CCTGGGCCTTGGTGTT-GAC-3′; the *Taq*Man probe was 5′-CAGGGAGCAGTA CGTGGTCCGAGCT-3′. The forward primer for VEGFR2 mRNA was 5′-CACCACTCAAACGCTGACATGTA-3′ and the reverse primer, 5′-CCAACTGCCAATACCAGTGGAT-3′; the *Taq*Man probe was 5′-TGCCATTCCTCCCCCGCATC-3′. The forward primer for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was 5′-GAAGGTGAAGGTCG-GAGT-3′ and the reverse primer, 5′-GAAGATGGTGATGG-GATTTC-3′; the *Taq*Man probe was 5′-CAAGCTTCCCG-TTCTCAGCC-3′. Conditions for the one-step RT-PCR were as follows: 30 min at 48°C (stage 1, reverse transcription), 10 min at 95°C (stage 2, RT inactivation and Ampli*Taq* Gold activation) and then 40 cycles of amplification for 15 s at 95°C and 1 min at 60°C (stage 3, PCR). The expression of angiogenic mediators was quantified according to a method described elsewhere (Yamamoto *et al*, 2004). Briefly, the intensity of the reaction was evaluated from the quantity of total RNA in HeLa cells (ng) corresponding to the initial number of PCR cycles to reveal the linear increase in reaction intensity (threshold cycle) for each sample on a logarithmic standard curve. Data on the quantity of RNA (ng) for angiogenic mediators were normalised using the data for GAPDH in each sample by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Double immunostaining for angiogenic mediators and cell marker

To examine the distribution of angiogenic mediator expressing cells in the bone marrow of MDS cases, mouse monoclonal antibody against VEGF (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), polyclonal rabbit antibody against bFGF (Santa Cruz Biotechnology Inc.), mouse monoclonal antibody against TNFα (Serotec Ltd, Oxford, UK), or polyclonal rabbit antibody against TGFβ (Santa Cruz Biotechnology Inc.) were applied on 4 μm thick formalin-fixed tissue sections, after treatment with 0·3% hydrogen peroxide in methanol to delete endogenous peroxidase activity and 10% normal goat serum for blocking non-specific binding of the antibody. Sections were then incubated with goat anti-mouse or rabbit IgG antibody (Dako) followed by a sensitive peroxidase-conjugated streptavidin system (Dako) with DAB as the chromogen. Next, sections were treated with 0·1% trypsin in Tris buffer, 0·3% hydrogen peroxide in methanol, 10% normal goat serum, and then incubated with mouse monoclonal antibody for the macrophage-lineage cell marker anti-CD68 antibody (Dako). Sections were then incubated with goat anti-mouse IgG antibody followed by a sensitive peroxidase-conjugated streptavidin system with TrueBlue Peroxidase Substrate (KPL, Inc., Gaithersburg, MD, USA) as the chromogen.

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Statistical analysis

Statistically significant differences in the quantitative analysis of MVD and mRNA expression were calculated using Mann–Whitney's *U*-test when comparing non-paired samples and Wilcoxon's test when comparing the paired samples, MDS and MDS → OL. Significant correlations between the collated data were calculated using Spearman's rank correlation coefficient. *P*-values < 0·05 were considered significant.

Results

Bone marrow MVD in controls, MDS, MDS → OL, and de novo AML cases

To allow MVD measurement, bone marrow specimens from nine controls, 10 paired MDS and MDS → OL, and eight *de novo* AML patients were immunostained for FVIII and VEGFR2. Distribution of FVIII and VEGFR2 antigens clearly visualised the vascular endothelium, which was easily distinguishable from megakaryocytes and enabled measurement of the MVD (Fig 1). The pattern of microvessels viewed with immunohistochemical staining for FVIII Fig 1A–D and VEG-FR2 (Fig 1E and F) was consistent, regardless of which vascular-associated antigen was stained. The density of vessels was higher in MDS (Fig 1B) and *de novo* AML (Fig 1D and F) in contrast to the scarce staining in controls (Fig 1A) and MDS → OL (Fig 1C and E).

Statistical analysis of relative vascular area revealed significant differences among MVD of controls (median, 0·16; minimum–maximum, 0·01–0·24), MDS (0·22; 0·13–0·47), MDS → OL (0·07; 0·02–0·21) and *de novo* AML (0·39; 0·12–0·54) patients (Fig 2A). For simplicity, only the results of FVIII staining are shown because the results of VEGFR2 staining were almost the same as those of FVIII. Increased bone marrow vascularity was found in MDS (*P* < 0·05) and *de novo*

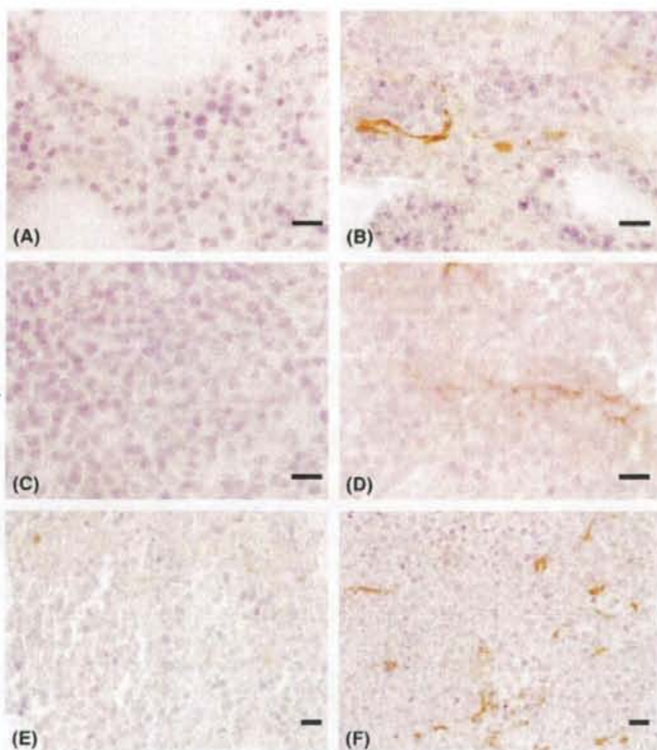

Fig 1. Immunohistochemical evaluation of vessels in the bone marrow of controls, MDS, MDS → OL, and *de novo* AML cases. Immunohistochemistry using anti-Factor VIII antigen identified blood vessels, clearly distinguishable from megakaryocytes (A, controls; B, MDS; C, MDS → OL; D, *de novo* AML; original magnification, 200×). Similarly, staining using anti-VEGFR2 clearly demonstrated higher density of blood vessels in *de novo* AML bone marrow (E) than that of MDS → OL bone marrow (F, original magnification, 100×). These images demonstrate the two antibodies produced staining of equal quality, identifying the vascular endothelium. Bars indicate 20 µm in each figure. Significant differences in vessel numbers were observed; controls and MDS → OL had the smallest amounts of vessels, *de novo* AML had numerous vessels; the numbers of vessels were intermediate in MDS.

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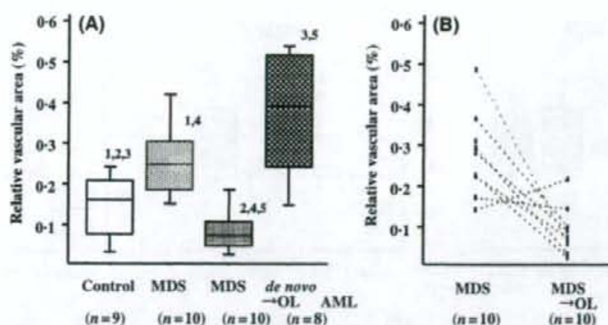

Fig 2. Comparison of the microvascular density (MVD) in the bone marrow of controls, MDS, MDS → OL, and *de novo* AML cases evaluated by image analysis using immunstaining for factor VIII antigen. (A) Box plot illustrating MVD measured, recorded as relative vascular area, in the bone marrow of controls, MDS, MDS → OL and *de novo* AML cases. Bars indicate 90% tile and 10% tile and boxes indicate 75% tile to 25% tile with lines of median values inside the boxes. Differences were significant between the MVD of controls and MDS ([1]$P < 0·01$), controls and MDS → OL ([2]$P < 0·05$), controls and *de novo* AML ([3]$P < 0·05$), MDS and MDS → OL ([4]$P < 0·001$), and MDS → OL and *de novo* AML ([5]$P < 0·01$). (B) Paired comparisons of the bone marrow MVD of patients with MDS and after transformation to OL. MVD was measured from paired bone marrow samples of patients with MDS and after transformation to OL. Note the decline in relative vascular area between MDS and MDS → OL, which was statistically significant ($P < 0·05$).

Table I. Quantitative analysis of angiogenic mediators in the bone marrow of controls, MDS, MDS → OL, and *de novo* AML cases determined by RT-PCR (the quantity of mRNA for angiogenic mediators normalised to that of GAPDH).

Mediator	Controls (n = 9)	MDS (n = 10)	MDS → OL (n = 10)	*de novo* AML (n = 8)
VEGF	0·66* (0·10–0·85)	0·54 (0·29–4·20)	0·73 (0·20–4·45)	1·13 (0·13–4·49)
bFGF	0·02 (0. 01–0·15)	0·09 (0·03–3·30)	0·04 (0·01–8·76)	0·64 (0·04–9·48)
TNFα	0·06 (0. 01–0·26)	0·24 (0·05–3·88)	0·11 (0·07–2·20)	1·95 (0·02–3·00)
HGF	0·05 (0. 01–0·21)	0·33 (0·06–4·59)	0·17 (0·04–3·25)	0·67 (0·06–62·9)
Ang-1	0·01 (0. 0002–0·09)	0·16 (0·02–3·27)	0·13 (0·01–2·51)	0·65 (0·01–6·41)
Ang-2	0·01 (0. 0004–0·25)	0·20 (0·03–4·42)	0·20 (0·03–2·84)	0·85 (0·02–20·8)
TGFβ	1·40 (0·14–6·62)	2·64 (1·00–12·4)	5·46 (1·03–25·2)	2·85 (0·16–62·9)
VEGFR2	0·14 (0·09–0·85)	0·15 (0·03–4·57)	0·10 (0·04–1·54)	0·28 (0·01–14·2)
TIE2	0·09 (0. 04–1·50)	0·15 (0·03–2·68)	0·10 (0·02–1·40)	0·42 (0·01–17·1)

*Values indicated are the median (range).

AML ($P < 0·05$) patients when compared with controls. Overall, MDS patients exhibited significantly lower MVD after transformation to OL ($P < 0·05$) and MDS → OL had lower MVD than *de novo* AML ($P < 0·01$). Median MVD values were higher in *de novo* AML than MDS, although the difference was not statistically significant. No significant difference was demonstrated between controls and MDS → OL. Pairwise comparison of MDS and MDS → OL samples clearly showed a significant reduction of MVD in MDS after transformation to OL (Fig 2B). As shown, nine of 10 MDS patients showed reduced MVD when OL developed. The remaining case showed a very small increase of MVD.

Quantitative analysis of the expression of mRNA for angiogenic mediators

To determine the gene expression intensity of angiogenic mediators VEGF, bFGF, TNFα, HGF, Ang-1, Ang-2 and TGFβ in the bone marrow specimens of controls, MDS, MDS → OL and *de novo* AML cases, real-time quantitative RT-PCR was performed (Table I). As shown in Fig 3A, VEGF expression was greater in *de novo* AML compared with controls ($P < 0·001$) and MDS ($P < 0·001$) but no other significant differences were found when comparing patient groups. The pro-angiogenic factors bFGF (Fig 3B), TNFα (Fig 3C), HGF (Fig 3D), and Ang-1 (Fig 3E) had significantly higher expression in MDS and *de novo* AML compared to controls, with higher expression in *de novo* AML compared with MDS. The gene expression of bFGF, TNFα and HGF was significantly higher in MDS patients prior to transformation to OL, and gene expression of bFGF in MDS → OL was significantly lower than that of *de novo* AML. The median value of gene expression of these factors was consistently greater in MDS → OL than controls, although this was not statistically significant.

To investigate whether the anti-angiogenic factors play a role in MVD, mRNA expression for Ang-2 and TGFβ was determined. As shown in Fig 3F, the expression pattern of Ang-2 in controls, MDS, MDS → OL and *de novo* AML was

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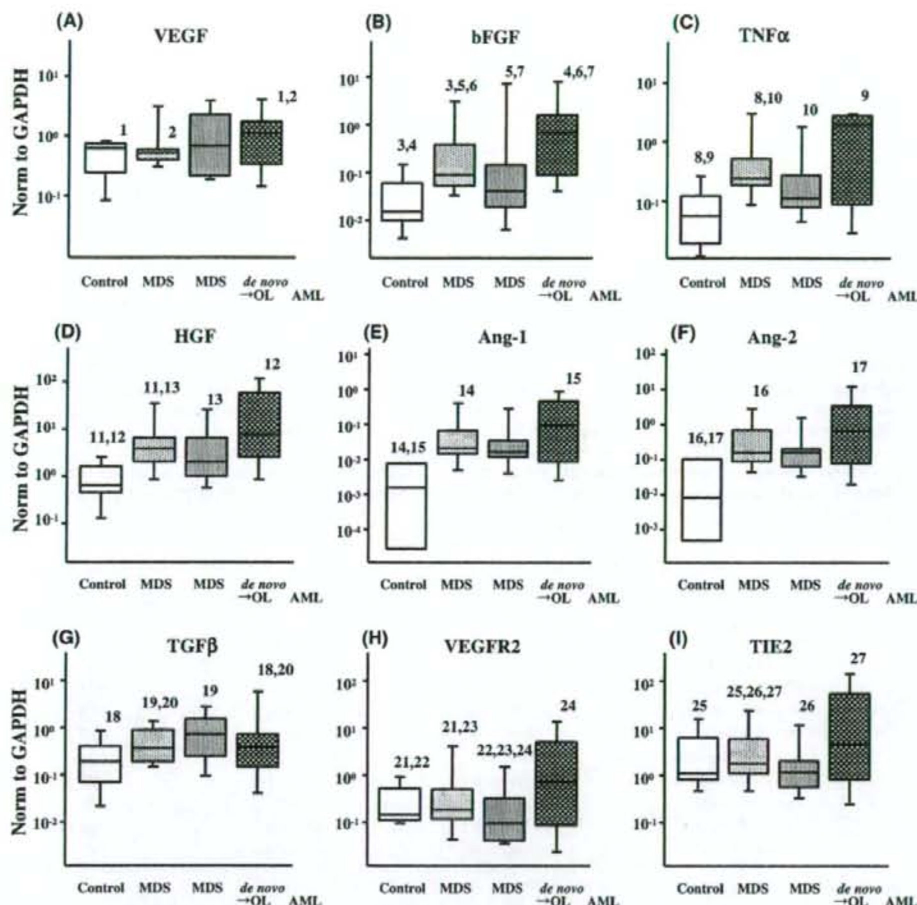

Fig 3. Expression of angiogenic mediators in the bone marrow of controls, MDS, MDS → OL, and *de novo* AML cases, as determined by RT-PCR. These box plots compare the levels of mRNA expression for VEGF (A), bFGF (B), TNFα (C), HGF (D), Ang-1 (E), Ang-2 (F), TGFβ (G) gene expression between controls, MDS, MDS → OL and *de novo* AML cases. Similarly, expression levels of receptors for angiogenic mediators, VEGFR2 (H) and TIE2 (I) were compared between controls, MDS, MDS → OL and *de novo* AML cases. Bars indicate 90% tile and 10% tile and boxes indicate 75% tile to 25% tile with lines of median values inside the boxes. Differences were significant between the expression levels in controls and *de novo* AML ([1]$P < 0·001$), and MDS and *de novo* AML ([2]$P < 0·001$) of VEGF, controls and MDS ([3]$P < 0·001$), controls and *de novo* AML ([4]$P < 0·01$), MDS and MDS → OL ([5]$P < 0·01$), MDS and *de novo* AML ([6]$P < 0·001$), and MDS → OL and *de novo* AML ([7]$P < 0·05$) of bFGF, controls and MDS ([8]$P < 0·05$), controls and *de novo* AML ([9]$P < 0·05$), and MDS and MDS → OL ([10]$P < 0·01$) of TNFα, controls and MDS ([11]$P < 0·01$), controls and *de novo* AML ([12]$P < 0·01$), and MDS and MDS → OL ([13]$P < 0·05$) of HGF, controls and MDS ([14]$P < 0·01$), and controls and *de novo* AML ([15]$P < 0·05$) of Ang-1, controls and MDS ([16]$P < 0·05$), and controls and *de novo* AML ([17]$P < 0·05$) of Ang-2, controls and *de novo* AML ([18]$P < 0·001$), MDS and MDS → OL ([19]$P < 0·01$), and MDS and *de novo* AML ([20]$P < 0·001$) of TGFβ. Expression levels of receptors for angiogenic mediators exhibited significant differences between controls and MDS ([21]$P < 0·001$), controls and MDS → OL ([22]$P < 0·001$), MDS and MDS → OL ([23]$P < 0·001$), MDS → OL and *de novo* AML ([24]$P < 0·001$) of VEGFR2, controls and MDS ([25]$P < 0·001$), MDS and MDS → OL ([26]$P < 0·01$), and MDS and *de novo* AML ([27]$P < 0·001$) of TIE2.

quite similar to that of Ang-1. TGFβ expression was in contrast to that of the pro-angiogenic factors, having significantly higher expression after transformation to OL than in MDS ($P < 0·01$), although, like the other pro-angiogenic factors, TGFβ showed increased expression in *de novo* AML compared with controls ($P < 0·001$) (Fig 3G).

Quantitative analysis of the expression of mRNA for receptors to angiogenic mediators

As shown in Fig 3H, VEGFR2 expression followed the trend of the other pro-angiogenic factors, with greatest expression in *de novo* AML, and higher expression in MDS compared with

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Table II. Correlations between MVD and expression intensities of angiogenic mediators.

	MVD	VEGF	bFGF	TNFα	HGF	Ang-1	Ang-2	TGFβ	VEGFR2	TIE2
MVD		NS	<0·05	<0·05	0·051	0·06	0·08	NS	<0·05	<0·05
VEGF	NS		<0·05	<0·05	NS	<0·05	<0·05	NS	0·055	<0·05
bFGF	<0·05	<0·05		<0·01	<0·05	<0·01	<0·01	NS	<0·05	<0·01
TNFα	<0·05	<0·05	<0·01		<0·05	<0·01	<0·01	NS	<0·05	<0·01
HGF	0·051	NS	<0·05	<0·05		<0·05	<0·05	NS	NS	<0·05
Ang-1	0·06	<0·05	<0·01	<0·01	<0·05		<0·001	NS	NS	<0·01
Ang-2	0·08	<0·05	<0·01	<0·01	<0·05	<0·001		NS	NS	<0·01
TGFβ	NS	NS	NS	NS	NS	NS	NS		NS	NS
VEGFR2	<0·05	0·055	<0·05	<0·05	NS	NS	NS	NS		<0·05
TIE2	<0·05	<0·05	<0·01	<0·01	<0·05	<0·01	<0·01	NS	<0·05	

*Values indicate the *P*-value calculated using Spearman's rank correlation coefficient. NS, not significant.

controls ($P < 0{\cdot}001$) and MDS → OL ($P < 0{\cdot}001$). Bone marrow samples from MDS → OL showed significantly lower VEGFR2 expression compared with those from *de novo* AML, MDS and controls ($P < 0{\cdot}001$). Similarly, TIE2 expression showed significant differences between controls and MDS ($P < 0{\cdot}001$), MDS and MDS → OL ($P < 0{\cdot}01$), and MDS and *de novo* AML ($P < 0{\cdot}001$) (Fig 3I).

Correlation of MVD and mRNA expression for angiogenic mediators and receptors

The immunohistochemical and PCR results identified a strong correlation between MVD and the expression of the receptors for pro-angiogenic mediators, VEGFR2 and TIE2 ($P < 0{\cdot}05$) (Table II). Gene expression of TNFα and bFGF also correlated with MVD ($P < 0{\cdot}05$). The expression of pro-angiogenic factors, VEGF, TNFα, HGF, bFGF, Ang-1 and Ang-2 showed strong correlation with one another (data not shown). The most significant correlations were observed between the expression of Ang-1 and Ang-2 ($P < 0{\cdot}001$) and Ang-1 and Ang-2 with receptor TIE2 ($P < 0{\cdot}01$). Expression of bFGF correlated with the expression of all pro-angiogenic factors ($P < 0{\cdot}01$ or $P < 0{\cdot}05$) and reversely correlated with TGFβ expression ($P < 0{\cdot}05$). TNFα showed strong correlations with bFGF, Ang-1, Ang-2 and TIE2 expression ($P < 0{\cdot}01$ or $P < 0{\cdot}05$). HGF showed no correlation with VEGF but correlated with the other pro-angiogenic factors ($P < 0{\cdot}05$). Interestingly expression of the receptors, TIE2 and VEGFR2, correlated ($P < 0{\cdot}05$). These results suggest the presence of an autocrine circuit influencing angiogenesis.

Localisation of angiogenic mediator producing cells in the bone marrow of MDS cases

The bone marrow environment is regulated by stromal cells, including endothelial cells and macrophage-lineage cells. Macrophage-lineage cells play a very crucial role in regulating haematopoiesis in MDS bone marrow (Kitagawa *et al*, 1997). To determine whether the cells expressing angiogenic mediators in MDS bone marrow were macrophage-lineage or not, double immunostaining for cell marker, CD68 and angiogenic mediators was performed. As shown in Fig 4, VEGF (A) and TGFβ (G) antigens were localised to CD68-negative cells, indicating that these molecules were mainly produced by haematopoietic cells in MDS bone marrow. By contrast, bFGF (C) and TNFα (E) were localised to CD68-positive macrophage-lineage cells, although bFGF was also localised to CD68-negative cells. When OL developed, some CD68-negative haematopoietic cells remained positive for VEGF antigen (B). However, angiogenic mediators, such as bFGF (D) and TNFα (F) positive cells, reduced in number, while TGFβ antigen was expressed in the blasts of OL bone marrow (H).

Discussion

The present study demonstrated a significant increase of MVD in *de novo* AML patients compared with controls, as previously demonstrated (Padro *et al*, 2000). The MVD in MDS was higher than in controls but lower than found in *de novo* AML and confirmed the results of Pruneri *et al* (1999), who proposed this implied angiogenesis was linked to the progression of MDS to OL. However, the present study clearly showed a decline in MVD upon transformation to OL from MDS, and showed that the MVD of MDS → OL was significantly lower than that of *de novo* AML. Gene expression of the angiogenic mediators also highlighted significant differences between MDS → OL and *de novo* AML, showing that, like MDS, *de novo* AML had higher expression of pro-angiogenic factors than MDS → OL.

Morphologically, blasts in MDS → OL and *de novo* AML patients were very similar; both groups are classified as FAB type AML M2, however, significantly different angiogenic activity has been demonstrated between the two groups. These findings indicate that the bone marrow microenvironment, and thus pathogenic events, in these diseases differs, as the clinical features, karyotypic abnormalities and responses to chemotherapy are different. The transformation of MDS to OL would be a multi-step, complex process. Our evidence suggests

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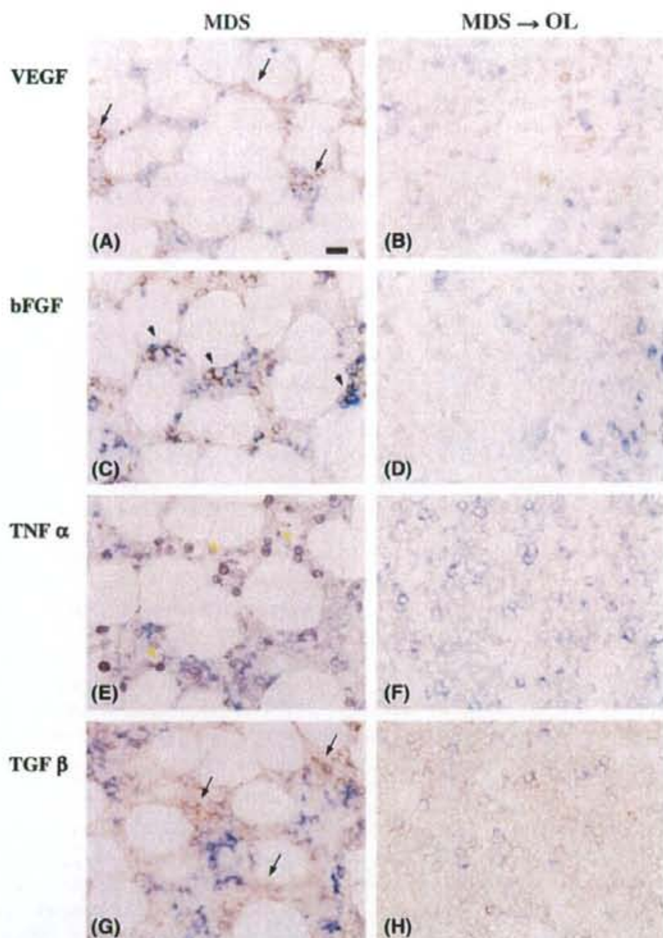

Fig 4. Immunohistochemical localisation of VEGF (A,B), bFGF (C,D), TNFα (E,F), and TGFβ (G,H) in the bone marrow of MDS cases at the time of initial diagnosis (MDS) and after evolution of OL (MDS → OL) (same magnification). Double immunostains for each mediator and the macrophage-lineage cell marker, CD68 in the bone marrow of MDS (A,C,E,G) and MDS → OL (B,D,F,H) are shown. Double immunostaining revealed positive reactions for each angiogenic mediator (brown) and the cell marker (blue). The bar in (A) indicates 20 μm. Note that VEGF- or TGFβ-positive cells in MDS bone marrow were CD68-negative (A and G, arrows), while bFGF or TNFα positive cells were also positive for CD68 antigen (C and E, arrowheads), although some bFGF positive cells were CD68-negative.

that, like *de novo* AML, MDS has increased angiogenesis; however, MDS → OL appears not to be as dependent upon angiogenesis as *de novo* AML, so there must be a point in the transformation of MDS to OL where disease evolution becomes independent of angiogenesis or resistant to angiogenic factors and no longer requires increased vascularity for increased proliferation and clonal expansion. This might be related to the chemotherapy-resistant nature of blasts in MDS → OL. Furthermore, reduced MVD could be an obstacle to the delivery of therapeutic drugs to the bone marrow environment. We suggest that anti-angiogenic drugs, i.e. targeted molecular therapy, would display differing efficacy between *de novo* AML and leukaemia secondary to MDS.

Angiogenic growth factors include VEGF, bFGF, TNFα, TGFβ and HGF. VEGF and bFGF are the strongest inducers of angiogenesis, synthesised in leukaemic and myeloma cells as well as fibroblasts, immune cells, osteoclasts, thrombocytes and megakaryocytes (Moehler *et al*, 2003). Aguayo *et al* (2000) demonstrated increased serum levels of VEGF, bFGF and HGF in *de novo* AML and MDS, and their association with increased MVD. The present study also showed that the gene expression of these factors was upregulated in the bone

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marrow, namely in the field of haematopoiesis in *de novo* AML and MDS.

Angiopoietin-2 can act as an anti-angiogenic factor by antagonising Ang-1 or, conversely, act under the influence of VEGF in a pro-angiogenic manner (Tait & Jones, 2004). The expression pattern of Ang-2 in MDS, MDS → OL, *de novo* AML and controls was quite similar to that of Ang-1. Their expression highly correlated with each other and the angiopoietin receptor TIE2, suggesting the presence of an autocrine circuit influencing angiogenesis. Furthermore, the correlation between the TIE2 and MVD confirmed the important role of angiopoietins in angiogenesis. Angiopoietin expressions also correlated with all the other angiogenic factors investigated, except TGFβ, indicating a close relationship amongst angiogenic factors, where expression of one influences another. Ang-1, Ang-2 and TIE2 were highly correlated with bFGF, a factor known to play a prominent role in angiogenesis (Bertolini *et al*, 2000) and one of the most commonly produced factors in tumours (Padro *et al*, 2000).

Interestingly, the expression of TGFβ was in contrast to the trend displayed by the other factors in this study, having increased expression in MDS → OL compared with MDS ($P < 0{\cdot}01$). TGFβ exerts bifunctional effects on endothelial cells *in vitro*; it can both stimulate and inhibit the proliferation of endothelial cells (Bertolino *et al*, 2006). Low doses of TGFβ stimulate endothelial proliferation, while high doses of TGFβ inhibit it. Recent studies have shown that TGFβ can regulate vascular homeostasis by balancing the signalling between two distinct TGFβ type I receptors [the endothelial-restricted activin receptor-like kinase (ALK) 1 and the broadly expressed ALK5 receptors] (Bertolino *et al*, 2006). The activation/regulation of these receptors has been shown to induce opposite effects on endothelial cell behaviour and angiogenesis. Angiogenesis can be roughly divided into an activation phase and a resolution phase. During the resolution phase, smooth muscle cells will be recruited to cover the new vascular tube and to inhibit the proliferation and migration of the endothelial cells. TGFβ would act as an inhibitory factor of endothelial proliferation directly or indirectly through the suppression of pro-angiogenic factors. Thus, the reduction of angiogenic activity/MVD in the bone marrow of MDS → OL might be attributable to the high expression of TGFβ.

In the present study, seven angiogenic factors and two receptors were studied and shown to have cooperative dynamics of expression for regulating angiogenesis in the bone marrow. However, numerous other angiogenic factors are known to be significant. When therapeutic implications are considered, the multiple proteins involved and their influence upon each other would complicate the effects of anti-angiogenic therapy. In addition, angiogenic factors often have multiple roles and are not specific surrogate markers of angiogenesis. VEGF, Ang-1 and Ang-2 act specifically upon endothelium, however, bFGF, HGF and TNFα are pleiotropic, targeting numerous cell types. As well as angiogenesis, TNFα contributes to apoptosis (Kitagawa *et al*, 1997; Sawanobori *et al*, 2003; Stifter *et al*, 2005) and bFGF is mitogenic for fibroblasts. Although angiogenesis is part of the pathogenic process in malignancy, it is also required physiologically (Bertolini *et al*, 2000). Therefore, although knowledge in this field is growing, anti-angiogenic therapy is not an easy cure, but does offer promise, as anti-angiogenic drugs target both the microenvironment and malignant cells directly (Aguayo *et al*, 2003), blocking angiogenic factors secreted by leukaemic and other cells, and breaking the autocrine and paracrine circuits which facilitate malignant growth. On the other hand, the promotion of angiogenesis in MDS → OL might even facilitate the delivery of anti-leukaemic drugs to the bone marrow environment. Further studies should determine whether anti-angiogenic therapy has differing efficacy between *de novo* AML and AML secondary to MDS, to clarify the role and importance of angiogenesis in these haematological malignancies.

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Rapid induction of IAP family proteins and Smac/DIABLO expression after proapoptotic stimulation with doxorubicin in RPMI 8226 multiple myeloma cells

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Abstract

We studied the expression dynamics of inhibitor of apoptosis protein (IAP) family members and Smac/DIABLO after *treatment* with doxorubicin in human multiple myeloma cell line RPMI 8226 and its doxorubicin-resistant variant DRR. Proapoptotic stimulation with doxorubicin rapidly induced the overexpression of mRNA as well as protein for IAPs in RPMI 8226 cells followed by a gradual decrease of their expression. Smac/DIABLO, which is known to neutralize IAPs, showed increased expression at the mRNA level after treatment; however, Western blot analysis revealed a slight decrease of the amount of protein. Immunoprecipitation analysis revealed the association of Smac/DIABLO with cIAP1 or XIAP after treatment with doxorubicin. In contrast to the RPMI 8226 cells, DRR cells did not undergo apoptosis in response to doxorubicin treatment. The DRR cells had higher levels of IAPs expression at the mRNA level and did not show a remarkable peak or decrease in the expression of mRNAs for cIAP1, cIAP2, XIAP, and survivin after treatment with doxorubicin. Furthermore, the expression of Smac/DIABLO mRNA was not up-regulated after treatment. These findings indicate that the suppression of IAPs expression by Smac/DIABLO shortly after proapoptotic stimulation might play a role in the mechanisms of apoptotic induction, and that the maintenance of high IAPs expression and low Smac/DIABLO expression after treatment might lead to the doxorubicin-resistance of multiple myeloma cells.
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Keywords: Apoptosis; Drug resistance; IAP; Smac/DIABLO; Multiple myeloma

Introduction

Inhibitor of apoptosis proteins (IAPs) were first identified in baculoviruses. All IAPs, including those from viruses as well as their cellular homologues in invertebrates and vertebrates, contain 1 to 3 baculovirus IAP repeat (BIR) motifs (Deveraux and Reed, 1999; Miller, 1999). In humans, eight kinds of IAPs, survivin, cIAP1, cIAP2, XIAP, NAIP, livin, apollon and ILP-2, have been identified (Abe et al., 2005; Yamamoto et al., 2004). The BIR domains of IAPs allow them to bind to and inhibit the proteases, caspases, that modulate the apoptotic destruction of cells. Although the exact biochemical mechanism by which these proteins suppress apoptosis is under debate, survivin, for example, is known to directly bind to and inhibit caspase-3 and -7, which act as terminal effectors in apoptotic protease cascades (Shin et al., 2001; Tamm et al., 1998). Survivin is widely expressed in fetal tissues, but its expression becomes restricted during development, and it is negligibly expressed in the majority of terminally differentiated adult tissues (Adida et al., 1998; Ambrosini et al., 1997). However, analysis of the differences in gene expression between normal cells and tumor cells has revealed that survivin is one of the genes that is most consistently overexpressed in tumor cells relative to normal tissues (Velculescu et al., 1999). In fact, survivin is prominently expressed in transformed cell lines and in many human cancers

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including hematopoietic cell tumors (Altieri and Marchisio, 1999).

The expression dynamics as well as the functional protein amount dynamics of IAP family proteins after stimulation for apoptosis-induction would have critical significance in regulating the apoptotic pathways of cells. Regarding the degradation of IAP family proteins, the ubiquitylation process has been a focus of attention recently. Another zinc-binding motif of IAPs, the RING domain, binds E2 ubiquitin-conjugated enzymes (UBCs). This enables RING-domain-containing proteins to recruit an E2 and catalyse the transfer of ubiquitin from the E2 to a substrate (Vaux and Slike, 2005). Such ubiquitylation might target IAPs or other IAP-interacting proteins for degradation, or might specifically change their activity. Thus, the degradation of not only IAPs themselves but also the associated proteins would be followed by complicated outcomes that should be controlled by much more complicated mechanisms.

Smac/DIABLO is also an important molecule that regulates the function of IAPs. The Smac/DIABLO protein resides in the mitochondria of healthy cells, and is released upon apoptotic stress with similar kinetics to cytochrome *c* (Du et al., 2000; Verhagen et al., 2000). Although the mechanism of Smac/DIABLO release has not been entirely resolved, this protein has been demonstrated to bind all of the IAPs tested to date (Liston et al., 2003). Smac/DIABLO can bind to the BIR domain of IAPs, thereby interfering with either caspase-3/-7 or caspase-9 inhibition.

Several chemotherapeutic drugs are known to down-regulate IAP family protein and mRNA expression and to cause caspase activation and apoptosis in human cancer cells (Tyagi et al., 2003; Wittmann et al., 2003). However, many types of cancer cells do resist chemotherapeutic induction of apoptosis in clinical situations as well as under various in vitro conditions. Thus, the effects of apoptosis-inducing drugs on the actual induction of apoptosis and the expression dynamics of various apoptosis-associated molecules in the apoptosic signaling pathways are complicated and still controversial. In the present study, we determined the expression dynamics of IAPs and Smac/DIABLO from the very early period after treatment with doxorubicin. The expression of IAPs exhibited up-regulation just after treatment with doxorubicin, followed by a gradual decrease in association with over-expression of Smac/DIABLO. Mechanisms regulating the up- and down-regulation of the expression of IAPs should provide clues to explaining the chemotherapy-resistant nature of cancer cells and to developing novel strategies to down-regulate anti-apoptotic molecules in human cancers. The implications of these findings regarding the drug resistance of cancer cells and their clinical significance are discussed.

Materials and methods

Cell lines

The establishment and characterization of the human multiple myeloma (MM) cell line RPMI 8226 was previously described (Dalton et al., 1986). The cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and routinely maintained in RPMI 1640 medium (Sigma, St Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Daiichi Seiyaku, Tokyo, Japan), 1% (v/v) penicillin at 100 units/ml (Invitrogen, Carlsbad, CA), and 1% (v/v) streptomycin at 100 units/ml (Invitrogen). We also generated the doxorubicin resistant variant of RPMI 8226 cell line, designated DRR, according to a previously described method (Dalton et al., 1986).

Induction of apoptosis by chemical agents

Doxorubicin hydrochloride (Wako, Tokyo, Japan) was used for inducing apoptosis in RPMI 8226 as well as DRR culture cells. Cells were treated with doxorubicin at the concentrations of 2, 5, and 10 μM in the culture medium described above.

Identification of apoptotic cells

To identify apoptotic cells by terminal deoxytransferase (TdT)-mediated dUTP nick end labeling (TUNEL), an *in situ* cell death detection kit, fluorescein (Boeringer Mannheim, Mannheim, Germany) was used as described previously (Kitagawa et al., 1998). Briefly, cells were collected before treatment and 0, 5, 1, 2, 3, and 6 h after treatment with doxorubicin, fixed with 4% paraformaldehyde for 20 min, washed with PBS and treated with 0.1% sodium citrate–0.1% Triton X-100 (Sigma) for 2 min. After washing with PBS, cells were mixed with FITC-dUTP and TdT at 37 °C for 60 min. Then, the TUNEL-positive cells were analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Preparation of RNA and quantitative assay for mRNA expression of IAP family proteins and Smac/DIABLO using TaqMan RT-PCR

RNA was extracted from RPMI 8226 culture cells, non-treated (NT) cells and doxorubicin-treated (2, 5, and 10 μM) cells at 0.5, 1, 2, 3, and 6 h after treatment using Trizol (Invitrogen) according to the manufacturer's directions. For quantitative RT-PCR, fluorescent hybridization probes and the TaqMan PCR Core Reagents Kit with AmpliTaq Gold (PerkinElmer Cetus, Norwalk, CT) were used with the ABI Prism 7900HT Sequence Detection System (PerkinElmer, Foster City, CA). Oligonucleotides used as specific primers and TaqMan probes for the IAP family proteins, Smac/DIABLO and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized at a commercial laboratory (PerkinElmer Cetus). The primers and TaqMan probes were as follows. The sequence for the forward primer for survivin mRNA was 5′-TGCCTGGCAGCCCTTTC-3′ and that for the reverse primer was 5′-CCTCCAAGAAGGGCCAGTTC-3′; for the TaqMan probe it was 5′-CAAGGACCACCGCATCTCTACATTC-3′. For cIAP1 mRNA, the sequence for the forward primer was 5′-CAGCCTGAGCAGCTTGCAA-3′ and that for the reverse primer it was 5′-CAAGCCACCATCACAACAAAA-3′; for the TaqMan probe it was 5′-TTTATTATGTGGGTCGCAATGATGATGTCAAA-3′. For cIAP2 mRNA, the sequences of the forward and reverse primer were 5′-TCCGTCAAGTTCAAGCCAGTT-3′ and 5′-TCTCCTGGGCTGTCTGATGTG-3′; respectively, and the sequence for the TaqMan probe was 5′-CCCTCATC-TACTTGAACAGCTGCTAT-3′. The forward and reverse sequences for NAIP mRNA were 5′-GCTTCACAGCGCATCGAA-3′ and 5′-GCTGGGCGGATGCT-TTC-3′; respectively, while the sequence for the TaqMan probe was 5′-CCATTTA-AACCACAGCAGAGGCTTTAT-3′. The sequence of the forward primer for XIAP mRNA was 5′-AGTGGTAGTCCTGTTTCAGCATCA-3′ and that for the reverse primer was 5′-CCGCACGGTATCTCCTTCA-3′; the sequence for the TaqMan probe was 5′-CACTGGCACGAGCAGGGTTTCTTTATACTG-3′. The sequence of the forward primer for Livin mRNA was 5′-TCTTCCACACAGGCCATCAG-3′ and that for the reverse primer was 5′-GTCCCCGCGCTTCCA-3′; the sequence for the TaqMan probe was 5′-ACAAGGTGAGGTGCTTCTTCTGCTAT-3′. The sequence of the forward primer for apollon mRNA was 5′-GCCGA-AGGATAGCGATCAG-3′ and that for the reverse primer was 5′-GCCCGGAA-GACGAAGAAA-3′; the sequence for the TaqMan probe was 5′-GTTGCGGCTCAACCTCCACCTATC-3′. The sequence of the forward primer for ILP-2 mRNA was 5′-CAGACTTCATTGCCGAGAGAAATCA-3′ and that for the reverse primer was 5′-CAGATTTTACAAAGCTTCTCCTCTTG-3′; the sequence for the TaqMan probe was 5′-CCCTGAAGAGCCGCTAAGGCGTCT-3′. The sequence of the forward primer for Smac/DIABLO mRNA was 5′-

GCTGGAAACCACTTGGATGAC-3′ and that for the reverse primer was 5′-TGCATATCAAACTGGCGCA-3′; the sequence for the TaqMan probe was 5′-CAGTTGGTCTTTCAGAGATGGCAGCAGA-3′. Finally, the forward primer sequence for GAPDH mRNA was 5′-GAAGGTGAAGGTCGGAGT-3′ and that for the reverse primer was 5′-GAAGATGGTGATGGGATTTC-3′; the TaqMan probe sequence was 5′-CAAGCTTCCCGTTCTCAGCC-3′. The conditions for one-step RT-PCR were as follows: 2 min at 50 °C (Stage 1, reverse transcription), 10 min at 95 °C (Stage 2, RT inactivation and AmpliTaq Gold activation) and then 45 cycles of amplification for 15 s at 95 °C and 1 min at 60 °C (Stage 3, PCR). Data on the quantity of RNA (ng) for the IAPs and Smac/DIABLO were normalized using the data for GAPDH in each sample and quantitated according to a method described elsewhere (Yamamoto et al., 2004).

Western blot analysis for IAP family proteins and Smac/DIABLO and immunoprecipitaion

RPMI 8226 cells from each experimental group were suspended in RPMI 1640 medium containing 10% fetal bovine serum at a concentration of 6×10^6 cells/tube and pelleted. Cell lysates were prepared by incubating the pellets on ice for 15 min in 1 ml of a lysis buffer containing 10 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1% Nonidet P-40, 0.02% NaN_3, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 0.1% aprotinin 100 μM leupeptin, and 100 μM tosyl-L-phenylalanyl chloromethyl ketone (TPCK) (Sigma). Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). The whole cell lysate (50 μg) was subjected to 12.5% SDS-PAGE. Gels were transferred electrophoretically to nitrocellulose membranes (Schleicher and Schull, Dassel, Germany). The membranes were blocked in 10% skim milk in PBS, incubated with a rabbit polyclonal anti-cIAP1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Smac/DIABLO (Santa Cruz Biotechnology) or a mouse monoclonal antibody to XIAP (R&D Systems, Minneapolis, MN), and after being washed were incubated with a horseradish peroxidase-conjugated anti-goat or anti-mouse IgG antibody (Dakopatts, Glostrup, Denmark). To confirm the equivalent loading of protein in each lane, membranes were also incubated in polyclonal rabbit anti-actin antisera (Sigma Chemicals). Bands in the washed membranes were detected with an enhanced chemiluminescence (ECL) system (Amersham Life Science, Buckinghamshire, England) as described previously (Kitagawa et al., 1996, 2002).

For immunoprecipitation experiments, cell lysates which contained 100 μg of protein were incubated with antibody against Smac/DIABLO and protein A-Sepharose beads (Amersham Life Science, Buckinghamshire, England). The resulting immunoprecipitates (50–100 μg of protein) were analyzed for cIAP1 or XIAP as described above.

The densities of bands were measured by densitometric analysis with an ImageQuant scanning imager (Molecular Dynamics, Sunnyvale, CA). The relative intensities of the bands were calculated by comparing the density of the sample with that of the control.

Results

Induction of apoptosis in RPMI 8226 cells and DRR cells by doxorubicin

To detect the actual induction of apoptosis in RPMI 8226 cells by doxorubicin at the concentration of 2, 5 and 10 μM, TUNEL-positive cell ratios were determined at various times after treatment (non-treated (NT), 0.5, 1, 2, 3, and 6 h). As shown in Fig. 1A, the ratio was increased in a dose-dependent manner at each time point. The ratio showed a gradual increase when cells were treated with 5 and 10 μM of doxorubicin, while the ratio was rather stable until 3 h after treatment with 2 μM doxorubicin. In contrast, DRR cells showed only a slight increase of TUNEL-positive cell ratio even after treatment with 10 μM doxorubicin (Fig. 1B).

Expression of mRNA for IAP family proteins determined by real-time quantitative PCR in RPMI 8226 cells and DRR cells after treatment with doxorubicin

To quantitate the mRNA expression levels of IAP family proteins in RPMI 8226 cell line cells and doxorubicin-resistant DRR cells, real-time quantitative RT-PCR was performed using samples treated with 2, 5, and 10 μM of doxorubicin or non-treated cells (NT), 1, 2, 3, and 6 h after treatment. Three samples at each point were examined for IAPs expression. For simplicity, error bars were not shown in the figures. As shown in Fig. 2A, most of the IAPs exhibited a peak of expression at 1 or 2 h after treatment with 2 or 5 μM doxorubicin in RPMI 8226 cells. The expression gradually decreased thereafter by 6 h. The levels of the induction of overexpression of cIAP1, cIAP2, survivin and apollon were higher,

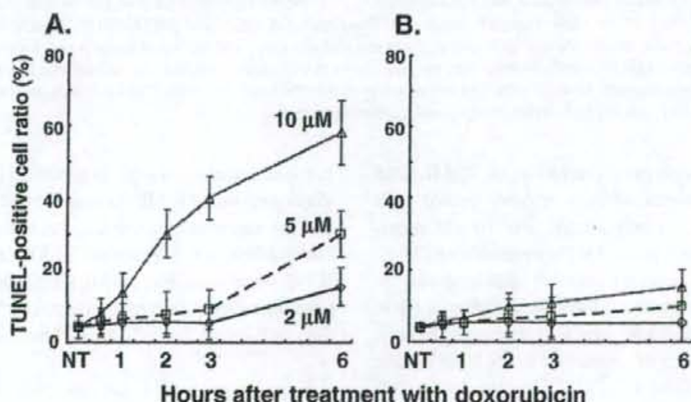

Fig. 1. Apoptotic cell ratio after treatment with doxorubicin determined by TUNEL method in doxorubicin-sensitive RPMI 8226 cells (A) and -resistant DRR cells (B). The solid line (o–o) indicates the ratio cells were treated with 2 μM doxorubicin, the dashed line, 5 μM doxorubicin (□–□) and the dotted line, 10 μM doxorubicin (△.....△). Error bars indicate standard deviation of the data in three samples at each point. Note that the apoptosis was induced in a dose-dependent and basically in a time-dependent manner in RPMI 8226 cells. By contrast, the apoptotic cell ratio was much lower in DRR cells.

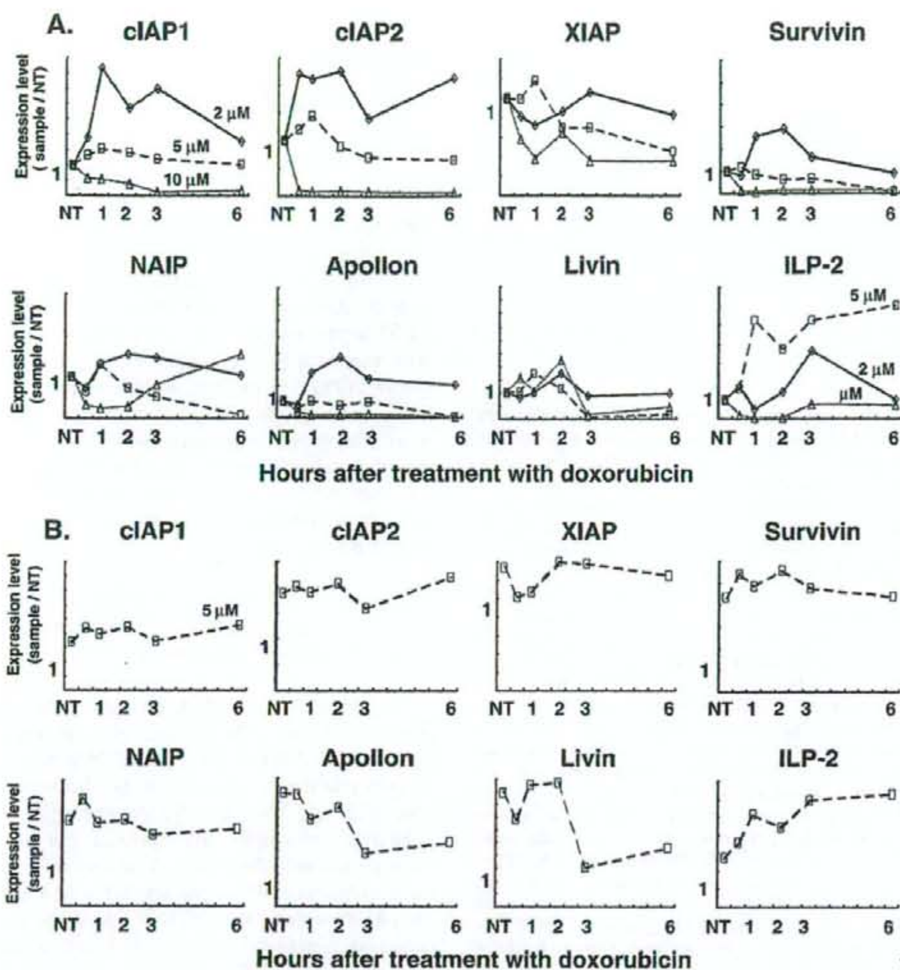

Fig. 2. Expression dynamics of mRNA of IAP family proteins in RPMI 8226 (A) and DRR cells (B) determined by the quantitative RT-PCR analysis. The expression levels of IAPs were determined in non-treated (NT) samples, and samples at, 0.5, 1, 2, 3, and 6 h after treatment with doxorubicin. The values are indicated as the ratio [IAP expression of RPMI 8226 cells or DRR cells after treatment (samples)/IAP expression of non-treated (NT) RPMI 8226 cells]. The solid line (◇—◇) indicates the value when cells were treated with 2 μM doxorubicin, the dashed line, 5 μM doxorubicin (□--□) and the dotted line, 10 μM doxorubicin (△.....△). The data were obtained from three samples at each point but for simplicity, error bars were not shown in the figures. Note that most IAPs showed increased expression forming a peak at 1 to 3 h after treatment with doxorubicin in RPMI 8226 cells. The expression gradually decreased thereafter. The DRR cells showed higher expression of IAPs than RPMI 8226 cells before treatment (NT) and the high levels of expression continued thereafter.

namely, the transient peaks were more prominent, in RPMI 8226 cells treated with 2 μM doxorubicin than in those treated with 5 μM. By contrast, RPMI 8226 cells treated with 10 μM doxorubicin did not show prominent peaks for the expression of IAPs but instead showed a gradual decrease from 1 h after treatment.

The levels of mRNA expression for IAPs were higher in DRR cells than RPMI 8226 cells before treatment with doxorubicin (non-treated samples: NT). The levels of expression in DRR cells were about 3 to 4 fold higher than those in RPMI 8226 cells. In contrast to the expression dynamics of IAPs in RPMI 8226 cells after treatment with doxorubicin, DRR cells exhibited minimal changes in expression of cIAP1, cIAP2, XIAP, survivin, NAIP and ILP-2 and continues to express high levels of the mRNAs for these IAPs at 6 h after treatment with doxorubicin (5 μM) (Fig. 2B). However, the expression of NAIP showed a small peak of expression at 0.5 h and the expression of apollon and livin was reduced by 3 and 6 h after treatment with doxorubicin. Although the expression levels of ILP-2 were similar in RPMI 8226 and DRR cells (5 μM doxorubicin), other IAPs exhibited much higher expression in DRR cells than in RPMI 8226 cells throughout the observation period.

Expression of mRNA for Smac/DIABLO determined by real-time quantitative PCR after treatment with doxorubicin

Next, to quantitate the mRNA expression of Smac/DIABLO in RPMI 8226 cells, real-time quantitative RT-PCR was performed

using the same samples as described above. As shown in Fig. 3A, Smac/DIABLO exhibited transient overexpression at 1 to 2 h after treatment with 2 or 5 μM doxorubicin in RPMI 8226 cells. Similar to the changes in IAPs, the expression levels of Smac/DIABLO were higher in cells treated with 2 μM doxorubicin than in those treated with 5 μM doxorubicin. The expression gradually decreased thereafter with 5 μM treatment, but was maintained at a high level until 6 h with 2 μM treatment. By contrast, treatment with 10 μM doxorubicin did not induce any changes of expression of mRNA for Smac/DIABLO in RPMI 8226 cells.

In DRR cells, the expression of Smac/DIBLO was almost twice as high as that in RPMI 8226 cells (non-treated samples: NT). In contrast to the remarkable up-regulation of the mRNA expression of Smac/DIABLO in RPMI 8226 cells, the DRR cells did not show a remarkable change of the expression of Smac/DIABLO after treatment even with 2 μM doxorubicin (Fig. 3B).

Expression of IAP family proteins and Smac/DIABLO determined by Western blotting after treatment with doxorubicin

To determine the dynamics of protein expression of IAPs and Smac/DIABLO after treatment with doxorubicin, Western blot analyses were performed using cell lysate from RPMI 8226 cell samples treated with 2, 5, and 10 μM of doxorubicin and NT, 0.5, 1, 2, 3, and 6 h after treatment. As shown in Fig. 4, cIAP1 and XIAP exhibited the elevated expression at 1 to 3 h after treatment with 2 μM doxorubicin. However, treatment with 5 μM doxorubicin induced overexpression at 0.5 to 2 h, and treatment with 10 μM doxorubicin induced overexpression at 0.5 h after treatment. The expression gradually decreased thereafter. By contrast, the expression of Smac/DIABLO showed a gradual decrease from 0.5 h after treatment with 2, 5, and 10 μM doxorubicin.

Interactions of IAPs with Smac/DIABLO in response to doxorubicin-treatment

To test whether IAPs actually interact with Smac/DIABLO in response to doxorubicin-treatment, lysates from RPMI 8226 cells treated with doxorubicin were immunoprecipitated with anti-Smac/DIABLO antibody and then the precipitates were immunoblotted with antibody against cIAP1 or XIAP. As shown in Fig. 5, co-precipitation of Smac/DIABLO and cIAP1 as well as Smac/DIABLO and XIAP was observed in the samples 0.5 to 2 h after treatment with doxorubicin. Taken together with the data from Figs. 3 and 4, these findings show that the expression of Smac/DIABLO was induced at the mRNA level after treatment with doxorubicin, and then the produced protein was recruited and bound to IAPs to form a IAP-Smac/DIABLO complex resulting in the rapid disappearance at the protein level.

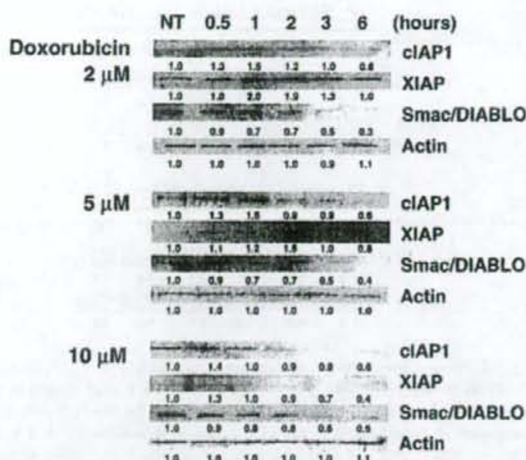

Fig. 4. Immunoblotting for cIAP1, XIAP, Smac/DIABLO in RPMI 8226 cells. Cell lysate (50 μg) from non-treated (NT) samples, and samples at, 0.5, 1, 2, 3, and 6 h after treatment with doxorubicin was used for this assay. Actin protein levels of each sample are shown to confirm that the amounts of samples loaded were almost equal. The relative intensities of bands were measured by densitometry (NT in each protein as the control, 1.0) and indicated under the photos of the gels. Note the transient increase of cIAP1 and XIAP expression in doxorubicin-treated RPMI 8226 cells in contrast to the gradual decrease of Smac/DIABLO expression after treatment with doxorubicin.

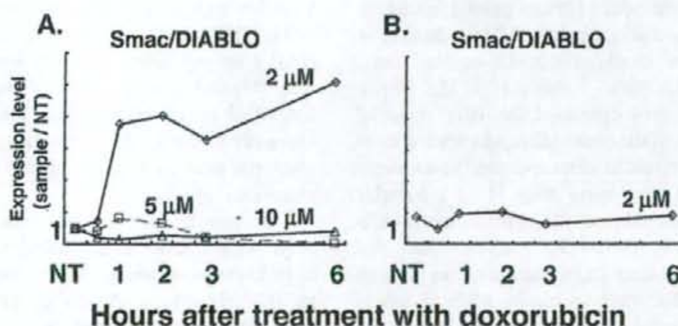

Fig. 3. Expression dynamics of mRNA of Smac/DIABLO in RPMI 8226 (A) and DRR cells (B) determined by the quantitative RT-PCR analysis. The expression levels of Smac/DIABLO were determined in non-treated (NT) samples, and samples at, 0.5, 1, 2, 3, and 6 h after treatment with doxorubicin. The values are indicated as the ratio [IAP expression of RPMI 8226 cells or DRR cells after treatment (samples)/IAP expression of non-treated (NT) RPMI 8226 cells]. The solid line (○—○) indicates the value when cells were treated with 2 μM doxorubicin, the dashed line, 5 μM doxorubicin (□--□) and the dotted line, 10 μM doxorubicin (△.....△). The data were obtained from three samples at each point but for simplicity, error bars were not shown in the figures. Note the increased expression of Smac/DIABLO in RPMI-8226 cells after treatment with 2 μM doxorubicin, while the expression did not show a remarkable increase in DRR cells.

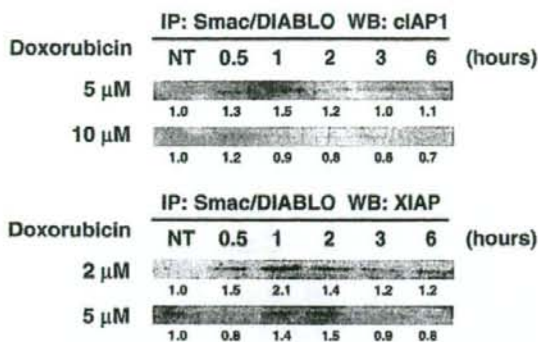

Fig. 5. Co-immunoprecipitation analysis for Smac/DIABLO and IAPs (cIAP1 or XIAP) in RPMI 8226 cells NT, 0.5, 1, 2, 3 and 6 h after treatment with doxorubicin (5 and 10 μM for cIAP1 and 2 and 5 μM for XIAP). Up-regulated association of Smac/DIABLO and cIAP1 was demonstrated 0.5 to 1 h after treatment with 2 or 5 μM doxorubicin and a significant association of Smac/DIABLO and XIAP was demonstrated 1 to 2 h after treatment with 2 or 5 μM doxorubicin. In RPMI cells treated with 10 μM doxorubicin, Smac/DIABLO and IAPs did not show the remarkable association.

This could explain why the protein analysis of Smac/DIABLO showed a gradual decrease after treatment with doxorubicin. These findings would also suggest that the decrease in the amount of IAP proteins might be related to the interaction between IAPs and Smac/DIABLO.

Discussion

In the present study we examined the possible role of IAPs and Smac/DIABLO in the regulation of apoptosis in RPMI 8226 multiple myeloma cells and the possible involvement of these proteins in the action of the chemotherapeutic agent doxorubicin and chemoresistance. Especially concerning the expression dynamics during the early period after doxorubicin treatment, we demonstrated a transient overexpression of IAPs. And the suppression of IAPs by Smac/DIABLO shortly after proapoptotic stimulation might play a role in the mechanisms of apoptotic induction. Previous studies have shown the down-regulation of IAPs expression after different types of proapoptotic stimulation such as treatment with doxorubicin, cisplatin, UV-irradiation, or TNF-α (Crnkovic-Mertens et al., 2003; Li et al., 2001; Notarbartolo et al., 2002, 2005; Yonesaka et al., 2006). However, the previous studies examined the later stage of expression dynamics in which the observation was started from 6–12 h after treatment. Our results demonstrated the transient up-regulation of IAPs at a very early stage (1 to 2 h) after proapoptotic simulation. This indicates the significance of IAPs dynamics at the very early period for understanding the biological mechanisms regulating IAPs expression as well as the interactions of IAPs with other molecules such as Smac/DIABLO. We have completed the same experiments using human T cell line, Jurkat cell line (data not shown). The results were almost the same with the present study. Thus, we prefer to speculate that the dynamics of IAPs and Smac/DIABLO after doxorubicin treatment was not specific for myeloma cells but for more general cells.

In the doxorubicin-resistant cell line DRR, we demonstrated the lower expression of Smac/DIABLO mRNA, resulting in the continuous overexpression of IAPs at the protein level, although the precise evaluation for the activation of Smac/DIABLO should be determined comparing the protein level both in the mitochondrial and cytosolic fractions. The expression dynamics of these molecules in DRR cells were thus characterized by a lack of down-regulation of IAPs and a lack of up-regulation of Smac/DIABLO after treatment with doxorubicin. Using the HL60 leukemia cell line and its multidrug resistant variant HL60R, HL60R cells were shown to overexpress the mRNAs of some IAPs as compared with HL60 (Notarbartolo et al., 2002). Doxorubicin or serum withdrawal strongly down-regulate survivin and XIAP mRNAs in HL60, while the same mRNAs are much less affected in HL60R cells. These results support the possibility that IAPs may play a role in the resistance to apoptosis of HL60R cells and further suggest that suppressor/neutralizers of IAPs such as Smac/DIABLO might have a significant role in controlling the drug-resistance of these cells. In the present study, we demonstrated the direct association of Smac/DIABLO with IAPs 1 to 2 h after treatment with doxorubicin. Smac/DIABLO is known to neutralize IAPs and thus, facilitates the proapoptotic process after apoptotic stimuli (Galluzzi et al., 2006), although the relationships between IAPs expression and Smac/DIABLO release are far from being completely understood (Liu et al., 2004; Duckett, 2005).

It would also be important to clarify the mechanisms responsible for the transient up-regulation of mRNA/protein expression for IAPs in RPMI 8226 cells after proapoptotic stimuli. Insulin-like growth factor-1 (IGF-1) and interleukin-6 (IL-6) promote the proliferation of multiple myeloma cells. IGF-1 stimulates the sustained activation of NF-κB and Akt and up-regulates a series of intracellular anti-apoptotic proteins, including FLIP, survivin, cIAP-2 and XIAP. In contrast, IL-6 does not cause sustained NF-κB activation, induces less pronounced Akt activation, and increases the expression of only survivin (Mitsiades et al., 2002a). We previously demonstrated that TNF-α is present locally in the bone marrow microenvironment and is associated with the regulation of cellular proliferation/apoptosis in hematological diseases (Kitagawa et al., 1997). TNF-α induces NF-κB nuclear translocation, cIAP-1 and cIAP-2 up-regulation, and proliferation in multiple myeloma cells (Mitsiades et al., 2002b). Thus, the expression of IAP is controlled by complex cellular signals. Further study will be necessary to clarify the mechanism of IAP induction in multiple myeloma cells in response to proapoptotic stimuli, including chemotherapy.

Our research interests deal with possible strategies to overcome the resistance to drugs and apoptosis, possibly related to IAPs expression, which characterize tumors with poor prognosis (Notarbartolo et al., 2005). Using bone marrow samples from patients with multiple myeloma, we have demonstrated that IAPs expression correlates with poor outcome in association with chemotherapy-induced overexpression of multidrug resistance genes (Nakagawa et al., 2006). Thus, the functional inhibition of specific IAPs may provide a rational basis for the development of novel therapeutic strategies. Using small

interfering (si)RNAs, which could efficiently block endogenous IAPs gene expression, HeLa cells were analyzed to test whether blockade of livin would actually be effective for inducing apoptosis in tumor cells. Silencing of livin was associated with caspase-3 activation and a strongly increased apoptotic rate in response to different proapoptotic stimuli, such as doxorubicin, UV-irradiation or TNF-α (Crnkovic-Mertens et al., 2003). Similarly, siRNA targeting survivin sensitized lung cancer cells with mutant p53 to doxorubicin (Yonesaka et al., 2006).

Here we demonstrated that proapoptotic stimulation induced the transient up-regulation of IAPs expression. This fact also indicated that cellular machineries such as the Smac/DIABLO system should work for the rapid down-regulation of the IAPs expression. Further studies should clarify the mechanisms responsible for IAPs as well as Smac/DIABLO regulation and also the regulation of down-stream molecules such as caspases in multiple myeloma cells and provide a tool for blocking the rapid induction of IAPs expression after proapoptotic stimulation with chemical agents. The highly preserved expression of IAPs and lower Smac/DIABLO expression might cooperate or interact to produce the doxorubicin-resistant conditions such as those in DRR cells. These results suggest that the DRR cells might lack the mechanisms for down-regulating IAPs and up-regulating Smac/DIABLO. Thus, to treat chemotherapy-resistant multiple myeloma cells like DRR, a novel chemotherapeutic strategy should be considered for targeting IAPs and enhancing the Smac/DIABLO system using IAP antagonists mimicking Smac/DIABLO, small-molecule BIR inhibitors, antisense oligonucleotides targeting IAPs (Mizukawa et al., 2006; Schimmer and Dalili, 2005; Wright and Duckett, 2005) or RNA interference of IAPs (Kashkar et al., 2006) in combination with proapoptotic chemotherapy.

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Interleukin (IL)-4 promotes T helper type 2-biased natural killer T (NKT) cell expansion, which is regulated by NKT cell-derived interferon-γ and IL-4

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Summary

CD1d-restricted natural killer T (NKT) cells can rapidly produce T helper type 1 (Th1) and Th2 cytokines and also play regulatory or pathological roles in immune responses. NKT cells are able to expand when cultured with α-galactosylceramide (α-GalCer) and interleukin (IL)-2 in a CD1d-restricted manner. However, the expansion ratio of human NKT cells is variable from sample to sample. In this study, we sought to determine what factor or factors are responsible for efficient *in vitro* expansion of NKT cells from various inbred mouse strains. Although the proportion of NKT cells in the spleen was nearly identical in each mouse strain, the growth rates of NKT cells cultured *in vitro* with α-GalCer and IL-2 were highly variable. NKT cells from the B6C3F1 and BDF1 mouse strains expanded more than 20-fold after 4 days in culture. In contrast, NKT cells from the strain C3H/HeN did not proliferate at all. We found that cell expansion efficiency correlated with the level of IL-4 detectable in the supernatant after culture. Furthermore, we found that exogenous IL-4 augmented NKT cell proliferation early in the culture period, whereas interferon (IFN)-γ tended to inhibit NKT cell proliferation. Thus, the ratio of production of IL-4 and IFN-γ was important for NKT cell expansion but the absolute levels of these cytokines did not affect expansion. This finding suggests that effective expansion of NKT cells requires Th2-biased culture conditions.

Keywords: natural killer T cell; interleukin-4; interferon-γ; glycolipid

Introduction

Mouse natural killer T (NKT) cells were initially identified as a T-cell subset that expresses NK cell receptors such as NK1·1, CD94 and Ly49.[1,2] The majority of NKT cells have the invariant T-cell receptor (TCR) α-chain rearrangement Vα14-Jα18 and recognize antigens presented by CD1d, a non-classical major histocompatibility complex (MHC) class I molecule.[3,4] NKT cells are continuously sensitized by endogenous antigens so that they display an effector-memory phenotype (such as $CD62L^{low}$ $CD44^{high}$)[5–7] and rapidly produce large amounts of T helper type 1 (Th1) and Th2 cytokines when stimulated with lipid antigens such as α-galactosylceramide (α-GalCer) in a CD1d-dependent manner.[2,8] NKT cells are regarded as immunoregulatory because of their cytokine profile. Moreover, NKT cells are thought to play an important role in response to infectious agents and in pathological responses such as allergies or autoimmune disease. NKT cells are cytotoxic to various tumour cell lines via Fas-ligand-, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)- and/or perforin-dependent pathways,[9–12] and play a role in tumour surveillance.[13] NKT cells activated by interleukin (IL)-12 or α-GalCer sequentially activate natural killer (NK) cells by producing interferon (IFN)-γ and induce antitumour immune responses. This in turn inhibits tumour metastasis and can suppress solid tumour growth. In some studies, it has been suggested that this ability helps to induce tumour antigen-specific CD8 T cells, thereby making an additional contribution to the immune response to cancer.[14]

In humans, counterparts of mouse NKT cells have also been found to be responsive to α-GalCer, which induces them to secrete IL-4 and IFN-γ. In addition, they have been shown to be cytotoxic to tumour cells via two different mechanisms, a CD1d-dependent and a CD1d-independent mechanism.[15] Human NKT cells have the

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