Negative regulation of erythroblast maturation by Fas-L⁺/TRAIL⁺ highly malignant plasma cells: a major pathogenetic mechanism of anemia in multiple myeloma

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Multiple myeloma (MM) is associated with severe normochromic/normocytic anemia. This study demonstrates that the abnormal up-regulation of apoptogenic receptors, including both Fas ligand (L) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), by highly malignant myeloma cells is involved in the pathogenesis of the ineffective erythropoiesis and chronic exhaustion of the erythroid matrix. By measuring Fas-L and TRAIL in plasma cells and the content of glycophorin A (GpA) in erythroblasts from a cohort of 28 untreated, newly diagnosed patients with MM and 7 with monoclonal gammopathy of undetermined significance (MGUS), selected in relation to their peripheral hemoglobin values, results showed that both receptors occurred at high levels in 15 severely anemic MM patients. Their marrow erythropoietic component was low and included predominantly immature GpA⁺dim erythroblasts, in contrast with the higher relative numbers of mature GpA⁺bright erythroblasts observed in the nonanemic patients and those with MGUS. In cocultures with autologous Fas-L⁺/TRAIL⁺ myeloma cells, the expanded GpA⁺dim erythroid population underwent prompt apoptosis after direct exposure to malignant plasma cells, whereas erythroblasts from nonanemic patients were scarcely affected. The evidence that Fas-L⁺/TRAIL⁺ malignant plasma cells prime erythroblast apoptosis by direct cytotoxicity was also supported by the increase of FLICE in fresh immature GpA⁺dim erythroid cells, whereas ICE and caspase-10 increased in subsequent maturative forms. In addition, GATA-1, a survival factor for erythroid precursors, was remarkably down-regulated in fresh erythroblasts from the severely anemic patients. These results indicate that progressive destruction of the erythroid matrix in aggressive MM is due to cytotoxic mechanisms based on the up-regulation in myeloma cells of Fas-L, TRAIL, or both. It is conceivable that the altered regulation of these receptors defines a peculiar cytotoxic phenotype that drives the progression of aggressive MM.

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Introduction

Defective erythropoiesis leading to chronic normochromic/normocytic anemia is common in the progression of multiple myeloma (MM). Its main features are hypoferrremia in the presence of adequate iron stores and inappropriate erythropoietin (EPO) production. Several negative regulators of erythropoiesis are involved in the pathogenesis of defective erythroid maturation. The erythropoietic precursors are acted on by apoptosis inducers, namely, transforming growth factor (TGF)–β1, tumor necrosis factor (TNF)–α, interferon (IFN)–γ, and Fas ligand (Fas-L). These proapoptogenic factors are physiologically involved in modulating the maturation of erythroid cells and are up-regulated in the anemia of chronic disorders, as well as in many solid tumors and hematologic malignancies, including MM. Malignant plasma cells can also add to the progressive destruction of the erythroid matrix by direct Fas-L-mediated cytotoxicity.

Recent work by independent groups has emphasized the role of the Fas/Fas-L system in restraining erythroid maturation. Pro-erythroblasts at the prebasophilic/basophilic stage are susceptible to apoptosis because of their high sensitivity to Fas stimulation, whereas mature erythroblasts are refractory to the autotoxic lysis induced by excess Fas-L. That Fas exerts a prominent role in the control of erythropoiesis is also supported by additional studies, showing that erythroblasts treated with TNF-α or IFN-γ or both strongly trigger apoptosis in response to the CH11 anti-Fas monoclonal antibody (mAb) as prototypic agonist ligand of the receptor. Furthermore, mouse embryos lacking FADD, a cytoplasmic Fas-associated domain involved in the sequential activation of downstream caspases, and undergo lethal erythroid accumulation, and TRAIL, the TNF-related apoptosis-inducing ligand, displays structural and functional similarities with Fas-L, and acts through FADD in the homeostatic regulation of erythropoiesis. However, in contrast with the inhibitory effect promoted by Fas-L in early erythroid precursors, TRAIL is apparently effective during the intermediate stage of erythroblast maturation when a mild to moderate presence of glycophorin A (GpA) is detectable in erythrocyte cells.

We have recently shown that Fas-L overexpression by myeloma cells reflects their high degree of malignancy, as assessed by their proliferation in the absence of interleukin-6 (IL-6) as well as their unresponsiveness to Fas stimulation by the CH11 mAb. These clones contribute to anemia in MM, because they exert a major cytotoxic effect on the narrow erythrocyte matrix by presenting...
Fas-L to immature erythroblasts.9 We hypothesize that erythropoietic failure also results from progressive depletion of mature erythroblasts as an effect of the inability of their precursors to proceed to mature forms in the presence of cytotoxic myeloma cells. This pathway then leads to a compensatory expansion of erythroblasts as an effect of the inability of their precursors to mature. The erythroid failure also results from progressive depletion of mature erythroblasts.20

In this study we have evaluated the occurrence of the cytotoxic phenotype in malignant plasma cells from a cohort of patients with MM. In the majority of those with severe anemia and active disease, these cells greatly inhibited erythroid maturation.

Patients, materials, and methods

Patients

Twenty-eight patients with MM stages I to IIIb2 were selected from a larger population such that 2 groups were constructed: those with peripheral hemoglobin of 9 g/dL or less (group A) and those with a value of 11.5 g/dL or higher (group B), to compare those with severe anemia to those with borderline or normal erythropoiesis. All patients were newly diagnosed and were included in the study before receiving any chemotherapy, to avoid the potential interference of cytotoxic drugs in cellular responses. Seven patients with monoclonal gammapathy of undetermined significance (MGUS) and normal erythropoiesis were also included. The study was approved by the Ethical Committee of the University of Bari. Informed consent was obtained from all individuals.

Bone marrow phenotyping

Bone marrow cells were freshly typed by double-fluorescence analysis in a FACScan (Becton Dickinson, Mountain View, CA). Phenotypic differentiation of erythroblasts from plasma cells was first assessed by detection of GA21 and CD38, respectively, using fluorescein isothiocyanate (FITC)–conjugated mAbs (Immunotech, Marseille, France, and Becton Dickinson, San Jose, CA). In addition, the expression of both Fas-L and TRAIL was measured in CD38− cell populations. The biotinylated IgG1k from clone NOK-1 (Pharmingen, San Diego, CA) treated with phycoerythrin (PE)–conjugated streptavidin was used for Fas-L, whereas the expression of TRAIL was assessed by a rabbit polyclonal antiserum (Alexis, Vinci, Italy) in association with a goat PE-conjugated secondary antibody.

Erythroid cell maturation was investigated by measuring the fluorescence intensity of GP a expression. Enriched erythroblast populations (2.5×105) were obtained by magnetic cell sorting of bone marrow, using anti-GpA–conjugated microbeads, which resulted in purities for GpA− cells of approximately 90% (Miltenyi Biotech, Bergisch Gladbach, Germany). Aliquots of these cells were then incubated with the PE-conjugated anti-GpA mAb (Immunotech) for semiquantitative flow cytometry evaluation of GpA. The analysis was completed with the Cell-Quest software (Becton Dickinson), which defines fluorescence intensity as dim, intermediate, and bright. These differential levels of GpA expression have been associated with the immature, semimature, and mature erythroblast stages of erythroid development.11

The expression of Fas, Fas-L, and TRAIL and its effector receptors DR4 and DR5 was also measured with respect to concurrent GpA content by double-fluorescence flow cytometry11 on isolated erythroblast populations. Fas was detected by the ZB4 IgG1 mAb (Immunotech), whereas rabbit and goat antisera were used for DR4 and DR5 molecules, respectively, and in all instances FITC-conjugated secondary antibodies were used for their measurement. In addition, erythroblasts were evaluated for the expression of GATA-1, namely, the transcription factor necessary for the terminal differentiation of the erythroid precursors.23,24 Aliquots of those cells were incubated with a rat anti-GATA-1 mAb (Santa Cruz Biotechnology, Santa Cruz, CA) and further treated with the FITC-conjugated secondary antibody prior to flow cytometry.

Cocultures of erythroblasts with myeloma cells

Mixed cultures of erythroblasts and plasma cells were set up to investigate the cytotoxic potential of both Fas-L+ and TRAIL+ myeloma cells. Similarly to the erythroblast preparations, malignant plasma cells were enriched by magnetic sorting with the anti-CD138 (syndecan-1)-25,26 mAb (Miltenyi Biotech). This method was successful in providing excellent purity (>96.5%) of the myeloma cells used for coculturing. The cultures were initiated using both EPO and IL-6, as reported.9 Briefly, 2 to 5×10⁵ fresh erythroblasts were incubated overnight in 24-well plates at 37°C in the presence of plasma cells at 1:1 ratio. Erythroblasts were cultured with autologous myeloma cells, and each coculture was prepared in parallel to measure the erythroblast apoptosis differentially induced by Fas-L, TRAIL, or both.

To measure the effect of Fas-L, myeloma cells were inhibited in their TRAIL expression by a 90-minute pretreatment with 10 μg/mL polyclonal anti-TRAIL antiserum (Alexis) and then added to the erythroblast preparations. Parallel functional assays were carried out to confirm the efficacy of the anti-TRAIL treatment, showing almost complete (91.3%) neutralization of the TRAIL-related cytotoxicity. This cytotoxicity was also assessed on further aliquots of myeloma cells, which were first incubated with 10 μg/mL of the recombinant Fas-Fc chimeric protein (Alexis) to inactivate Fas-L and then cocultured with the erythroblasts. The ability of this soluble isoform of Fas to saturate the binding site of Fas-L, leading to its functional impairment, had already been demonstrated.8 Subsequently, apoptosis of the erythroblast populations was measured.

Apoptosis

Apoptosis in cocultured erythroblasts was evaluated in comparison with control unstimulated cells from each patient, using a double-fluorescence assay that detects the outer leaflet exposure of phosphatidylserine by FITC–conjugated anti-fluorescein antibody and with the PE-labeled anti-GpA mAb (Immunotech). The extent of erythroblast apoptosis was then measured in the relevant GpA− subset with the Cell-Quest software.

The occurrence of apoptosis in cocultured erythroblasts was also investigated by direct examination under a fluorescence microscope. Briefly, the cells were first treated with the PE-conjugated reagent to measure the erythroblast apoptosis differentially induced by Fas-L, and TRAIL, and TRAIL expression by a 90-minute pretreatment with 10 μg/mL polyclonal anti-TRAIL antiserum (Alexis) and then added to the erythroblast preparations. Parallel functional assays were carried out to confirm the efficacy of the anti-TRAIL treatment, showing almost complete (91.3%) neutralization of the TRAIL-related cytotoxicity. This cytotoxicity was also assessed on further aliquots of myeloma cells, which were first incubated with 10 μg/mL of the recombinant Fas-Fc chimeric protein (Alexis) to inactivate Fas-L and then cocultured with the erythroblasts. The ability of this soluble isoform of Fas to saturate the binding site of Fas-L, leading to its functional impairment, had already been demonstrated.8 Subsequently, apoptosis of the erythroblast populations was measured.

Caspase activity

Several experiments were performed to assess caspase expression and activation in fresh erythroblasts. The constitutive expression of FLICE (caspase-8), ICE (caspase-1), and caspase-10 was measured by double-fluorescence analysis. FLICE was detected by a mAb (Biosource, Camarillo, CA), and ICE and caspase-10 by rabbit polyclonal antisera (Biosource and Prosci, Poway, CA, respectively). Although most reagents had been optimized by their manufacturers for immunoblotting methods, their reliability was checked by flow cytometry assay.9 Cells were incubated with the PE-anti-GpA mAb, then fixed, permeabilized, and separately treated with the PE-conjugated annexin-V (Boehringer Mannheim, Milan, Italy) and with the PE-labeled anti-GpA mAb (Immunotech). The extent of erythroblast apoptosis was then measured in the relevant GpA− subset with the Cell-Quest software.

Statistical analysis

Differences between means of groups of data were calculated using the nonparametric Mann-Whitney test.

Results

Expression of Fas-L and TRAIL and inhibition of erythroblast maturation in anemic patients

Table 1 shows the distribution of both anemic (group A) and nonanemic (group B) patients in parallel with the measurement of
from patients with MM or MGUS.

As expected, patients with advanced stage III disease were related to relative erythroblast numbers. Overall, the pattern in MGUS was similar to that of group B ($P = .63$). These data corroborated the evidence that the expression of Fas-L (and TRAIL as well) by malignant plasma cells is inversely related to relative erythroblast numbers.

Both plasma cells (CD38$^+$) and erythroid precursors (GpA$^+$) in the bone marrow. Fas-L and TRAIL expression by plasma cells as well as analysis of the distribution of GpA$^+$ subsets of enriched erythroblast populations are also included and compared with the results from patients with MGUS.

As expected, patients with advanced stage III disease were prevalent in group A, compared with group B. Furthermore, the expression of GpA$^+$ cells in bone marrow aspirates varied greatly in patients with MM. The erythroblast population (mean percent of GpA$^+$ cells) was less than half in group A compared to group B ($P = .1666$). By contrast, group A patients had significantly lower percentages of GpA$^+$ cells than group B and MGUS ($P < .0001$). Both receptors were concurrently overexpressed by myeloma cells, especially in patients A-01, A-06, and A-14. By contrast, CD38$^+$ cells in both group B and MGUS patients had much lower expression of both receptors, and there were no significant differences between the 2 receptors ($P \geq .1$).

No difference was recorded for GpA$^+$ dim cells in both group B and MGUS ($P = .1$). Erythroblasts obtained by magnetic sorting were distributed in relation to the fluorescence intensity of GpA expression as dim, intermediate, and bright flow cytometric pattern, namely, immature, partially differentiated, and mature erythroblasts, respectively. Numbers are percent of positive cells.

Hb indicates hemoglobin; ND, not done.

The 28 patients with MM were selected in relation to their peripheral hemoglobin levels (group A, $= 9$ g/dL; group B, $= 11.5$ g/dL). Erythroblasts obtained by magnetic sorting were distributed in relation to the fluorescence intensity of GpA expression as dim, intermediate, and bright flow cytometric pattern, namely, immature, partially differentiated, and mature erythroblasts, respectively. Numbers are percent of positive cells.
in both instances), whereas no difference was found between these 2 groups (P = .61), in keeping with their intact erythropoiesis as assessed by normal blood hemoglobin levels.

Figure 1 shows the GpA fluorescence intensity patterns of bead-purified erythroblasts from representative patients. As can be seen, the erythroid population from patients A-01 and A-14 was predominantly GpA\textsuperscript{+dim}, whereas that of patients B-02 and B-12 was mostly composed of GpA\textsuperscript{+bright} cells. This inverse pattern of the immature and mature erythroblast subsets correlated with the actual level of red cell production in these patient groups and suggested that the defective erythroblast maturation in group A was related to the greater expression of apoptogenic receptors by malignant plasma cells.

In the next set of experiments, the fluorescence intensity of GpA was correlated with the expression of a number of membrane receptors involved in the erythroblast apoptosis pathway, Fas-L and TRAIL are physiologically expressed by normal erythroblasts as their maturation progresses, presumably to control erythropoiesis, whereas their coreceptors, namely, Fas and DR molecules, are functional during early or intermediate maturation.\textsuperscript{10,17}

This analysis was completed in all subjects from groups A and B, with the exception of patients A-10 and B-06 because of insufficient GpA\textsuperscript{+} cells, and showed a substantial difference in the mean percent cells expressing these receptors (Figure 2). Apopto-
gen receptors, namely, Fas, DR4, and DR5, increased in expression during maturation in both groups. However, the levels of Fas were significantly higher in all subsets of GpA\textsuperscript{+} cells from group A (P < .05 in all instances). The highest Fas expression (73.4% ± 8%) occurred within the GpA\textsuperscript{+dim} population from group A compared with only 37.1% ± 5% in group B. In addition, though at lower levels, Fas was also up-regulated in both GpA\textsuperscript{+interm} and GpA\textsuperscript{+bright} erythroblasts from group A patients as compared to those of group B. The expression of DR4 and DR5 was also different, in that GpA\textsuperscript{+interm} erythroblasts from group A showed higher levels of both receptors than those from group B, whereas their expression was overall equivalent within the other cell populations.

By contrast, there were no differences between groups A and B in the occurrence of Fas-L and TRAIL, which were poorly expressed during the early maturative stages, but increased expression within the GpA\textsuperscript{+bright} population.

These results suggested that progression through erythroblast maturation in MM is regularly associated with a variable expression of phenotypic markers, namely, GpA, Fas-L, TRAIL, and their coreceptors. However, abnormal up-regulation of functional molecules, such as Fas and the receptors to TRAIL, is apparently predominant during both the early and the intermediate maturative steps of erythropoiesis, especially in anemic patients.

Apoptosis of erythroblasts from patients with MM in the presence of Fas-L\textsuperscript{+} /TRAIL\textsuperscript{+} myeloma cells

To resemble in vivo conditions, coculture experiments of erythro-
blasts with autologous myeloma cells were carried out to assess the susceptibility of erythroblasts to apoptosis in the presence of autologous Fas-L\textsuperscript{+} /TRAIL\textsuperscript{+} plasma cells. To obtain separate measurements of the effect of Fas-L or TRAIL, each coculture was set up in triplicate to include untreated plasma cells to determine the cumulative efficacy, plasma cells first TRAIL disabled by saturation with anti-TRAIL mAb to evaluate the effect of Fas-L, and plasma cells first Fas-L inactivated by soluble Fas to verify TRAIL activity. The effect of these prenested cell preparations, named TRAIL- or Fas-L-inhibited plasma cells, was compared with that of control erythroblast suspensions cultured in their absence. After coculturing, the cells were washed, treated with the
FITC-annexin-V and the PE-anti-GpA mAb, and then investigated by double-fluorescence flow cytometry. The analysis was completed in 8 cocultures using cells from group A patients (patients 01, 02, 04, 06, 07, 11, 12, and 14) and in 7 from group B (patients 02, 04, 05, 07, 10, 11, and 12).

Figure 3A shows mean values for erythroblast apoptosis in these cultures. Apoptosis was significantly higher in cultures from group A using untreated as well as TRAIL- or Fas-L–inhibited myeloma cells ($P < .02$ in all instances). Cultures from group B had no significant cytotoxicity of autologous plasma cells. However, a decline of these values was observed in cultures using inhibited malignant plasma cells, which still maintained a significantly higher level of erythroblast apoptosis than control erythroblast suspensions ($P < .02$). Control erythroblasts treated with either anti-Fas CH11 MoAb or recombinant TRAIL (Alexis) to resemble the effect of both apoptogenic receptors showed an apoptosis extent similar to that induced by either TRAIL-inhibited or Fas-L–inhibited plasma cells, respectively, whereas in parallel
In vivo activation of erythroblast apoptosis

Further experiments were designed to determine whether erythroblasts can be activated in vivo to apoptosis following their putative stimulation by malignant myeloma cells, by measuring the expression of cytoplasmatic proteases in freshly derived erythroblasts from several patients (A-01, A-04, A-06, A-07, A-12, A-14 and B-04, B-05, B-07, B-11). FLICE, ICE, and caspase-10 were investigated by double-fluorescence analysis and correlated with GpA intensity. Their expression varied in relation to the concurrent state of erythropoiesis (Figure 4A). FLICE was predominantly expressed (>82.0%) by the GpA<sup>dim</sup> cells from group A patients, thus confirming the expansion of the immature erythroid subset.

Although we measured the expression of the native proenzyme forms of the caspases, their dramatic accumulation was interpreted as presumably induced in vivo because the GpA<sup>dim</sup> cells from group A patients as well as the full erythroblast population from group B patients showed a moderate presence of FLICE<sup>+</sup> cells (20%-35%) that reflected its constitutive expression. By contrast, both ICE and caspase-10 were poorly expressed by GpA<sup>dim</sup> erythroblasts from all patients, and again increased remarkably within the GpA<sup>bright</sup> population of group A. The cellular content of both procaspases was significantly higher than in the group B GpA<sup>bright</sup> subset (P < .05 in both instances).

Figure 4B depicts the relative enrichment of caspase-positive cells within fresh erythroblasts from patients A-14 and B-07 in relation to their GpA levels. The GpA<sup>dim</sup> cells from patient A-14 showed the highest frequency of FLICE expression, suggesting that the biochemical transduction of death signals through this caspase was operative in vivo, in particular in immature erythroid cells (green). By contrast, both ICE and caspase-10 were largely expressed by the GpA<sup>bright</sup> erythroblasts (red) from both patients, though their maximum increase occurred in mature cells from patient A-14. Therefore, the high level of caspase expression in...
fresh erythroid cells from anemic patients supports the hypothesis
that activation of erythroblast apoptosis in vivo occurs via parallel
transduction pathways of death signals in relation to their matura-
tive steps; immature erythroblasts are more susceptible to Fas-
transduced apoptosis as a result of FLICE overexpression, whereas
the increase of both ICE and caspase-10 in GpA <sup>d</sup> cells
emphasizes their sensitivity to TRAIL as maturation progresses.

**Defective in vivo GATA-1 expression by erythroblasts from
anemic MM patients**

Because impaired erythropoiesis in severely anemic patients
was associated with a maturative arrest of erythroid precursors by the
malignant myeloma cells, experiments were carried out to assess
whether the expression of GATA-1, one transcriptional factor that
regulates erythroblast maturation, was concurrently disturbed.
Thus, the cellular content of GATA-1 was measured by double-
fluorescence analysis within each erythroblast population with
respect to its maturation in a number of patients from both groups
(A-02, A-05, A-09, A-11, A-14 and B-01, B-07, B-08, B-11).

Similar results were recorded within each group of patients.
Figure 5 depicts this analysis in representative patients (A-05 and
B-08) and shows the expected discrepancy in the relative distribu-
tion of their GpA <sup>d</sup> and GpA <sup>b</sup> erythroblast populations. The
immature erythroblast subset (R1) in patient A-05, in fact, was
greatly expanded (70%) to the detriment (<10%) of the mature
cell population (R2), whereas a lower percentage of immature cells
(33.5%) was detected in patient B-08. However, a substantial
diversity in GATA-1 expression was recorded between the 2
GpA <sup>d</sup> populations. The immature erythroblast subset in patient
A-05 was associated with a low fluorescence intensity of the
transcriptional factor (M1 peak: 76.1% of positive cells), whereas a
high fluorescence intensity (M2 peak) was detected in only a
minority of the GpA <sup>d</sup> erythroblasts (23.7% of positive cells). By
contrast, the immature erythroblasts from patient B-08 showed the
opposite pattern of GATA-1 content; the M1 peak included 37.3%
extent of positive cells, whereas the subset of immature cells with
high intensity of GATA-1 expression was considerably elevated (62.4%).

However, by measuring GATA-1 fluorescence intensity in
mature erythroblasts from both patients, a substantial equivalence
of data was observed because both the M1 and the M2 peaks
showed comparable results in both patients (M1, 41.8% versus
48.1%, and M2, 57.2% versus 51.7% in patients A-05 and B-08,
respectively). These findings suggested that a major defect of
GATA-1 expression was detectable in immature erythroblasts, in
particular in patients with severe anemia, and we hypothesize this
was associated with the cleavage of its native form induced by
Fas-L or TRAIL or both.  

**Discussion**

The present study addresses a novel mechanism of anemia in MM
and possibly also in other hematologic malignancies. Because
severe anemia associated with bone lesions defines the clinical
progression of MM, we explored the role of up-regulated apopto-
genic receptors exposed by highly malignant myeloma cells in the
destruction of the erythroid matrix. We measured the levels of both
Fas-L and TRAIL expression by bone marrow plasma cells from a
cohort of patients with untreated MM or MGUS, grouped in
relation to the occurrence of anemia, and found that their highest
intensity was detectable in severely anemic subjects with clinical
signs of active disease. The erythroblast population in their marrow
aspirates was constitutively depleted and mostly composed of
GpA <sup>d</sup> cells. This relative GpA <sup>d</sup> expansion was not detected in
myeloma patients without severe anemia, in that they exhibited both
GpA <sup>b</sup> and GpA <sup>d</sup> cells and their erythropoiesis was comparable
to that in MGUS patients.

In addition, coculturing these erythroblasts with autologous
myeloma cells resulted in marked erythroblast cytotoxicity induced
by either Fas-L or TRAIL or both presented by the malignant
plasma cells from the severely anemic patients with MM. The
initiator and executioner caspase <sup>39</sup> content of fresh erythroblasts
was also determined to assess whether these cytotoxic mechanisms
were operative in vivo. FLICE was greatly expressed by immature
erythroblasts in anemic patients and a concurrent increase of both
ICE and caspase-10 was detectable in their mature erythroid cells.
Erythropoiesis was also studied by measuring the content of
GATA-1 in each erythroblast subset. This transcriptional factor of
erythroid maturation was extremely down-regulated in the ex-
panded GpA <sup>d</sup> population of the anemic patients.

Taken together, these findings strongly suggest that in the
absence of any suppressive effect induced by chemotherapy,
several cytotoxic mechanisms are exerted by myeloma cells and are
responsible for chronic inhibition of erythropoiesis. Moreover, the
abnormal overexpression of apoptogenic receptors such as Fas-L or
TRAIL (or both) defines the major malignant phenotype associated
with progression of MM.
A persistent derangement of the cytokine network is thought to contribute to the defective erythropoiesis in hematologic malignancies, leading to chronic normochromic/normocytic anemia. TNF-β inhibits the formation of erythroid colony-forming unit in mice and decreased hemoglobin values in metastatic cancer patients treated with its recombinant form in a phase I trial. Increased TNF-β serum levels in both MM and other malignancies are thus suspected to down-regulate the maturation of erythroblasts by direct cytotoxicity, or by enhancing the susceptibility of their apoptosis receptors. IFN-γ secretion is also up-regulated in hematologic malignancies. This proinflammatory cytokine is believed to contribute to the progression of anemia in MM and in other cancers by up-regulating Fas in GpA erythroid precursors sensitive to those cell death activators including ICE, CPP32, and FLICE. However, the pathogenic role of these cytokines in the progression of MM is still controversial, because no correlation between IFN-γ or TNF-α levels and several variables of MM clinical activity was apparent in a previous study. The great expansion of annexin-V dim erythroblasts cultivated with Fas-L+ or TRAIL+ malignant plasma cells strongly suggests that the cytotoxic killing of erythroid cells was carried out via both Fas and the receptors to TRAIL, presumably up-regulated in vivo by those proinflammatory cytokines.

Our data indicate that the extensive damage to the erythropoietic process caused by Fas-L+/TRAIL+ myeloma cells and resulting in severe anemia occurs at different maturation phases. GpA dim erythroblasts, particularly at the basophilic stage, show maximal Fas sensitivity despite their poor responsiveness to TRAIL, though they express both DR4 and DR5 molecules as agonist receptors of this ligand. Conversely, GpA int dim erythroblasts, obtained in vitro by maturation of CD34+ hematopoietic progenitors with EPO, are susceptible to TRAIL-mediated apoptosis. Both subsets are driven to apoptosis by Fas-L+ and TRAIL+ GpA bright erythroblasts, which operate a negative regulatory feedback to inhibit excess maturation, whereas they are resistant to most apoptogenic ligands. Therefore, the ultimate stage of erythroid precursors sensitive to those cell death activators includes GpA int dim erythroblasts.

The expansion of GpA dim erythroblasts in the severely anemic patients with MM suggests that the enhanced apoptosis during maturation is the major event leading to defective erythropoiesis. In the presence of an excess of both Fas-L and TRAIL provided by the accumulation of malignant myeloma cells, maturation is suppressed at either the GpA dim or the GpA int dim stage. Accretion of GpA dim erythroblasts in group A patients may thus be a consequence of maturative arrest, whereas the few erythropoietic precursors that escape the Fas-L/TRAIL cytotoxicity of malignant myeloma cells complete their differentiation and become GpA bright erythroblasts. Figure 6 illustrates the hypothetical events that possibly lead to the maturative arrest of erythropoiesis and to the gradual destruction of erythroid productive capacity by Fas-L+/TRAIL+ highly malignant myeloma cells.

This interpretation is supported by the caspase content of fresh erythroblasts. The largest FLICE+ population was found in GpA dim erythroblasts from severely anemic patients, thus suggesting Fas pathway activation in vivo, whereas ICE and particularly caspase-10, which have been associated with the TRAIL pathway, were increased in subsequent maturation phases. The low to moderate expression of these caspases in erythroblasts from myeloma patients with efficient erythropoiesis emphasizes the substantial defect of Fas-L/TRAIL-mediated cytotoxicity in those cells.

Another interesting point of this study is the defective content of GATA-1 in erythroid precursors from anemic patients. This transcription factor promotes survival and is essential for the terminal differentiation of erythroblasts, because it binds regulatory motifs in globin genes and promotes the activation of other functional genes committed to the expression of EPO receptors, GpA, and transferrin receptor. Elegant studies by De Maria and coworkers have demonstrated that GATA-1 is promptly cleaved by several caspases, including FLICE, in CD34+ cell-derived erythroblasts, following their treatment with the agonist anti-Fas mAb from clone CH11, or with TRAIL. This cleavage may be supposed to lead to maturative arrest. Our evidence of a clear-cut defect of the native form of GATA-1 in the expanded GpA dim population (Figure 5) is in line with these studies and demonstrates that myeloma cells may also down-regulate GATA-1 by both Fas-L and TRAIL pathways, in addition to direct cytotoxic killing.

The median survival of patients with MM receiving conventional chemotherapy with melphalan plus prednisone is approximately 36 months. However, patients with aggressive disease are, or rapidly become, refractory to this treatment and show early expansion of myeloma cells. Despite years of intensive investigation, the molecular phenotype(s) of highly malignant plasma cells associated with the invasive evolution of MM have not yet been fully defined. Our studies emphasize the deregulated apoptosis in myeloma cells and
correlate the occurrence of specific phenotypic and functional derangements with their ability to proliferate and expand within the bone marrow to the detriment of other cells. The apoptogenic phenotype of plasma cells,\textsuperscript{45} as identified by both the abnormal overexpression of Fas-L\textsuperscript{3} and TRAIL and the deficient response to Fas stimulation, together with IL-6 insensitivity,\textsuperscript{19} may well be a marker of a high degree of malignancy and unrestraining progression.

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