Contribution of the induction of heme oxygenase-1 to etoposide-induced apoptosis in acute myeloblastic leukemia

Heme oxygenase-1 (HO-1) protects cells from cytotoxicity. It is induced by stressful stimuli. In the present study we show that induction of HO-1 during oxidative and nitrosative stress did not protect acute myeloblastic leukemia cells from etoposide-induced apoptosis and expression of HO-1 was not associated with etoposide resistance.

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Etoposide, a DNA topoisomerase II inhibitor, is an important anti-tumor agent. Etoposide resistance is a clinical problem, but its mechanisms are poorly understood. Etoposide may exert its cytotoxic activity by changing cellular redox state.[1-3] Heme-oxygenase-1 (HO-1) is presented as a cytoprotective enzyme that is induced in oxidative and nitrosative stress.[4,5] Theoretically, it might play some role in drug resistance. There is a report showing that etoposide -induced cytotoxicity is reduced in cells over-expressing HO-1.[6] We were interested in investigating whether HO-1 has any role in etoposide resistance in acute myeloblastic leukemia (AML).

The study was performed with two OCI/AML-2 subclones, etoposide-sensitive (ES) and etoposide-resistant (ER), developed in our laboratory from the parental cell line. Induction of HO-1 was analyzed by Western blotting. Cell death was determined by annexin V assay with propidium iodide. More than 90% of the dead cells were apoptotic, the remaining were necrotic.

After 24 hours' exposure of the subclones to 5.0 μM ETO, induction of HO-1 was observed in the ES subclone but not in the ER subclone (Figure 1A-B). At the same time, the percentage of dead cells was found to be 52±5 (n=3) in the ES subclone and 23±5 (n=3) in the ER subclone (Figure 2). Buthionine sulfoximine (BSO) induces oxidative stress by depletion of glutathione. In the ES subclone, the expression of HO-1 was higher in the presence of BSO + etoposide than in the case of either of the agents alone (Figure 1A). On the other hand, HO-1 was not induced in the ER subclone by these agents (Figure 1B). Furthermore, sodium nitroprusside, a nitric oxide donor and nitrosative stress-inducing agent, was observed to be a more powerful inductor of HO-1 in the ES subclone than in the ER subclone (Figure 1A-B). BSO + sodium nitroprusside caused a more significant increase in HO-1 than either of the agents alone in both subclones (Figure 1A-B). The most significant increase in HO-1 induction, however, was obtained with the combination of BSO, sodium nitroprusside + etoposide (Figure 1A-B). The experiment was repeated three times with comparable results. BSO and sodium nitroprusside as single agents did not cause any obvious increase in apoptosis in either of the subclones (Figure 2). BSO + etoposide caused an obvious increase in apoptosis in both subclones when compared to the effect of etoposide alone, the effect being more potent in the ES subclone than in the ER subclone (Figure 2). In contrast, SNP + etoposide did not cause any significant change in the apoptosis induced by etoposide alone (Figure 2). BSO + sodium nitroprusside did not cause apoptosis, and the addition of etoposide to them did not cause more apoptosis than the use of BSO + etoposide (Figure 2).

Our hypothesis was that HO-1 is induced more easily in the ER subclone than in the ES subclone and provides protection against etoposide-induced cell death in AML. This was not the case. On the contrary, the most marked induction of HO-1 was observed in the ES subclone, and in fact no induction of HO-1 was observed in the resistant subclone during etoposide exposure. This may support the idea that there was no need for HO-1 induction in the ER subclone, since other antioxidative systems were responding to the increased oxidative stress. This is supported by our previous studies on these subclones, which showed that the induction of mitochondrial manganese superoxide/dismutase and the higher basal glutathione and γ-glutamylcystine synthetase levels protected the ER subclone from etoposide-induced apoptosis compared to the ES subclone.[3,7] In the present study, combined use of BSO and etoposide increased the induction of HO-1 and apoptosis, especially in the

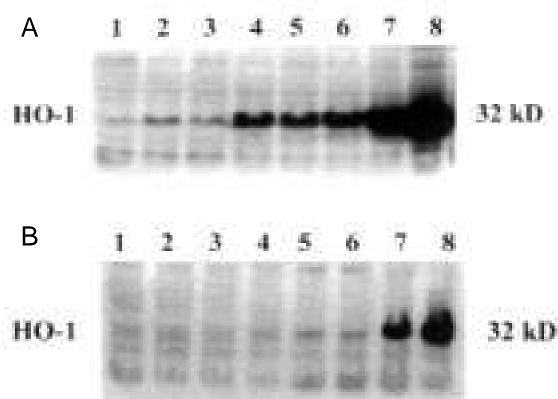

Figure 1. Representative gel electrophoreses of HO-1 expression on Western blotting after exposure of the cells to various stress-inducing agents. When appropriate, the cells were exposed first to 0.5 mM buthionine sulfoximine (BSO) and/or 1.0 mM sodium nitroprusside (SNP) for 24 hours, after which 5.0 μM etoposide (ETO) was added for another 24 hours. Cells without any exposure served as controls. In the studies, 100 mg of cell lysate protein were loaded on SDS-polyacrylamide gel electrophoresis and visualized by enhanced chemiluminescence and a specific anti-HO-1 antibody. 1= no exposure, 2= ETO, 3= BSO, 4= BSO + ETO, 5= SNP, 6= SNP + ETO, 7= SNP + BSO, 8= SNP + BSO + ETO. A. The etoposide-sensitive subclone. B. The etoposide-resistant subclone.

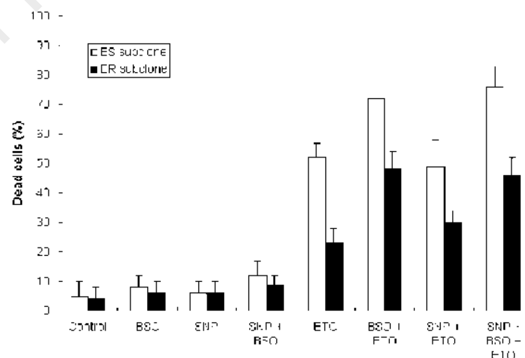

Figure 2. Cell death as percentages of controls in the etoposide-sensitive (ES) and resistant (ER) subclones after exposure of the cells to various agents as described in the legend to Figure 1. Cells without any exposure served as controls. Cell death (apoptotic and necrotic cells) was estimated by the annexin V assay with propidium iodide. The results are mean ± SD of three independent experiments.

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ES subclone. Thus, the induction of HO-1 was merely a sign of oxidative stress. Nitric oxide is a powerful inducer of HO-1,[8] and this was shown in the present study. However, it did not offer any protection against apoptosis induced by oxidative stress. Theoretically, nitric oxide may increase the induced apoptosis, since it may react with the superoxides formed during BSO/etoposide exposure, leading to peroxinitrate radical formation, which itself is toxic for many proteins and enzymes.[9] This was not perhaps the case in this study, however, since nitric oxide did not cause any increase in the apoptosis induced by the other agents.

In conclusion, HO-1 does not seem to confer etoposide resistance in AML cells and, its induction does not protect AML cells from etoposide-induced apoptosis.

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Expression of vascular endothelial growth factor and angiopoietin-2 in myeloma cells

Angiogenesis is important for myeloma. To understand the mechanism of angiogenesis, we analyzed vascular endothelial growth factor and angiopoietins, new angiogenic molecules, in purified myeloma cells at mRNA and protein levels. Co-expression of these factors was observed. We suggest that these molecules may be significant as prognostic factors.

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Angiogenesis is important for progression of hematologic malignancies including multiple myeloma (MM).[1,2] The most potent angiogenic factor is vascular endothelial growth factor $(VEGF)_{165}$.[3] It has been reported that the level of VEGF in the bone marrow plasma correlates with the activity of disease. Serum levels of VEGF were higher in a group of patients who showed poor response to chemotherapy.[4] However, it has not been clearly reported that VEGF is produced by myeloma cells.

Recently, it was reported that angiopoietins are essential for angiogenesis. Angiopoietin-1 induces phosphorylation of Tie-2, the common receptor of angiopoietin-1 and 2, and contributes to the recruitment and maintenance of an association between peri-endothelial supporting cells and endothelial cells, resulting in stabilization of vessel structures.[5] In contrast, angiopoietin-2 reduces interactions between supporting cells and endothelial cells, leading to production of new vessel sprouting.[6] It has also been reported that co-expression of angiopoietin-2 and VEGF co-ordinately promotes new vessel sprouting.[7] A correlation between the expression of angiopoietin-2 and angiogenesis has already been reported in certain types of cancer tissues[8] while the production of angiopoietins by myeloma cells is not known.

We determined expression of VEGF and angiopoietins in 39 samples from 34 patients (31 patients with MM, 2 with monoclonal gammopathies of undetermined significance (MGUS) and one with reactive bone marrow plasmacytosis). Plasma cells were purified by negative selection with anti-CD2, CD3, CD4, CD14, CD19, CD66b and glycophorin A-conjugated immunomagnetic beads. The primers utilized for the reverse transcriptase polymerase chain reaction (RT-PCR) are shown in Table 1. Immunohistochemical staining was also performed using LSAB Kit (Dako, Carpinteria, CA, USA) and human polyclonal antibodies against $VEGF_{165}$ (Neomarkers, Fremont, CA, USA) and angiopoietin-2 (KM202).[7]

Expression of $VEGF_{121}$ and $VEGF_{165}$ was detected in, respectively, 30 and 22 samples out of 36 bone marrow samples from patients with MM (Figure 1-A). Twenty-seven of 36 MM samples showed expression of angiopoietin-2 (Figure 1-A). Co-expression of $VEGF_{165}$ and angiopoietin-2 was detected in 18 MM samples, indicating that this is not a rare phenomenon in MM. Since VEGF and angiopoietin-2 were reported to work co-ordinately in angiogenesis, it is possible that these molecules induce angiogenesis in bone marrow of myeloma patients.[7] The two cases of MGUS and a case of reactive plasmacytosis did not show expression of $VEGF_{121}$ or $VEGF_{165}$, while low levels of angiopoietin-2 were detected (Figure 1-B). Absence of VEGF may be a characteristic of plasma cells in MGUS patients and plasmacytosis, indicating that VEGFs are important factors for malignant plasma cells. It could be speculated that plasma cells from MGUS or reactive plasmacytosis do not require as much neovascularization as myeloma cells because of their low proliferation. Further analysis of more cases is needed to clarify this hypothesis. Expression of angiopoietin-1 was not detected at all. The presence of $VEGF_{165}$ and angiopoietin-2 at protein level was confirmed by immunohistochemical staining in representative samples (Figure 1-C).

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