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Research Article

Induction of apoptosis and CD10/neutral endopeptidase expression by jaspamide in HL-60 line cells

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Abstract. Jaspamide (jasplakinolide) is a natural peptide isolated from marine sponges of *Jaspis* species and has fungicidal and growth-inhibiting activities. We characterized the jasplakinolide-induced loss of viability by programmed cell death in the HL-60 human promyelocytic leukemia cell line and found that this process was accompanied by neutral endopeptidase (NEP)/CD10 expression on the surface of the apoptotic cells. HL-60 cells do not normally express detectable amounts of NEP/CD10 on their surface or intracytoplasmically, but upon jaspamide treatment, CD10 was synthesized de novo, its expression being inhibited by cycloheximide pretreatment. Once synthesized, NEP/CD10 interfered with the jasplakinolide signal delivered to HL-60 cells. Inhibition of NEP/CD10 by the NEP inhibitor phosphoramidon or by an anti-CD10 monoclonal antibody significantly increased apoptosis induction. The appearance of CD10 on the cell surface was blocked by preincubation of the cells with the monocytic/macrophage-differentiating agents vitamin D_3 and phorbol 12-myristate 13-acetate, but not by the granulocytic differentiating agents retinoic acid or dimethyl sulfoxide. Moreover, in the promonocytic U937 and mature monocytic THP-1 cell lines, jaspamide induced apoptosis but not CD10 expression. In HL-60 cells, CD10 expression was partially but not totally blocked by the broad-spectrum caspase inhibitor benzyloxacarbonyl-Val-Ala-Asp-fluoromethylketone, indicating a connection between apoptosis induction and CD10 synthesis. Our findings suggest that the CD10 expression is related to the programmed cell death induction by jaspamide, and also with the process of granulocytic differentiation in HL-60 cells.

Key words. Apoptosis; caspase; jasplakinolide; myeloid leukemia; neutral endopeptidase.

Introduction

Jaspamide (jasplakinolide) is a natural cyclodepsipeptide with a 15-carbon macrocyclic ring isolated from marine sponges of the genus *Jaspis* (*Jaspis johnstoni*) [1] and from the marine sponge *Hemiastrella minor* [2]. The function of this peptide in the organisms from which it was isolated is unknown, but it has antifungal, insecticidal and antihelmintic bioactivity [1, 3, 4]. Jaspamide also has a strong antiproliferative activity against three human immortalized prostate carcinoma cell lines (PC-3, LNCaP and TSU-Pr1) [5] and against human Jurkat T cells [6].

In a recently published study [7], we described significant jaspamide-induced polyploidization in the HL-60 human promyelocytic/myeloblastic cell line at concentrations as low as 50 nM. During experiments, we observed that at higher concentrations of jaspamide, proliferation of HL-60 cells was totally inhibited, prompting us to initiate the present study to explore the antiproliferative activity of jaspamide on this leukemic cell line.

The common acute lymphoblastic leukemia-associated antigen CD10 was first defined serologically by Greaves et al. [8] and was thought to be specifically expressed on

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malignant cells from acute lymphoblastic leukemia patients. Subsequently, it was described on normal early human lymphoid progenitors within the bone marrow, fetal liver and thymus [9], on terminally differentiated granulocytes [10] and a variety of nonhematopoietic cell types including bronchial epithelial cells, cultured fibroblasts, bone marrow stromal cells, renal proximal tubular epithelial cells, breast myoepithelium, biliary canaliculi, fetal intestine and certain solid tumor cell lines [11], indicating that the antigen was not restricted to the lymphoid subset. Later, cloning, sequence analysis and functional studies identified CD10 as the cell surface enzyme neutral endopeptidase 24.11 [12], also known as metalloendopeptidase or enkephalinase, which was initially purified from the brush border of rabbit kidney proximal tubules [13].

Recently, CD10 expression was associated with programmed cell death induction in human T cells [14], so we decided to explore a possible link between apoptosis induction by jaspamide in the HL-60 cell line and neutral endopeptidase/CD10 expression, even if NEP/CD10 was reported to be virtually absent on these cells [10, 15]. In this study we report that jaspamide induced programmed cell death in HL-60 cells and that this apoptosis induction was accompanied by de novo synthesis of NEP/CD10. Our results indicate that the newly synthesized CD10 expression was related with the programmed cell death induction in HL-60 cells and with caspase activity, and also with the process of cellular differentiation in these cells.

Materials and methods

Reagents and antibodies

The jaspamide used in this study was purchased from Molecular Probes (Eugene, Ore.). A stock solution of 1 mM jaspamide was prepared in dimethyl sulfoxide (Sigma, St. Louis, Mo.) and stored at –20 °C. The murine anti-CD10 monoclonal antibodies (mAbs) used for flow cytometric analysis were the RPE-conjugated anti-CD10 (clone SS2/36, IgG_1 subclass) from Dako A/S (Glostrup, Denmark) and RPE-conjugated anti-CD10 (ALB1, IgG_1) from Immunotech (Coulter, Marseille, France). For control staining, a corresponding RPE-conjugated IgG_1 murine isotype was purchased from Becton Dickinson Immunocytometry Systems (San Jose, Calif.). The RPE-conjugated anti-CD19 (HIB19, IgG) was obtained from PharMingen International (Japan) and the RPE-conjugated anti-CD5 (L17F12, IgG_2) from Becton Dickinson. The FITC-conjugated mouse anti-human Bcl-2 oncoprotein (clone 124, IgG_1) was from Dako, and the PE-conjugated polyclonal rabbit anti-active caspase-3, anti-human Bax and anti-human Mcl-1 from PharMingen. For the rabbit unlabeled antibodies, a biotinylated second-step swine anti-rabbit mAb from Dako coupled with RPE-conjugated streptavidin was used. The broad-range spectrum caspase inhibitor benzyloxacarbonyl-Val-Ala-Asp-fluoromethylketone (z-vad.fmk) was purchased from Sigma. The neutral endopeptidase inhibitor phosphoramidon was obtained from Wako Pure Chemical Industries (Osaka, Japan). The protein synthesis inhibitor cycloheximide was purchased from Sigma-Aldrich (Milan, Italy). For detection of intracellular antigens, the Dako IntraStain fixation and permeabilization kit for flow cytometry was used. The transmembrane potentiometric fluorescent marker 3,3′-dihexyloxacarbocyanine (DiOC) was purchased from Molecular Probes. The FITC-conjugated rabbit polyclonal anti-poly (ADP-ribose) polymerase (PARP) cleavage site (214/215) was obtained from BioSource International (Camarillo, Calif.). The PE-conjugated Apo2.7 mAb (2.7A6A3, IgG_1) was purchased from Immunotech. The apoptosis membrane marker Annexin-V-Fluos FITC-labeled was obtained from Boehringer Mannheim (Germany). The counterstaining dyes for cell viability used together with annexin V FITC were propidium iodide from Calbiochem (La Jolla, Calif.) and 7-amino-actinomycin D (7-AAD) from Molecular Probes.

Leukemic cell lines

The HL-60 human promyelocytic/myeloblastic cell line [16] was purchased from the American Type Culture Collection (Rockville, Md.). The U937 promonocytic cell line [17] and THP-1 mature monocytic cell line [18] were purchased from Health Science Research Resources Bank (Osaka, Japan).

Cell culture and differentiation

HL-60 cells were cultured in RPMI 1640 medium (Nikken BioMedical Laboratory, Kyoto, Japan) supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, N. Y.), 2 mM L-glutamine and 1% antibiotics (penicillin 100 IU/ml, 100 µg/ml streptomycin and fungizone; Bio-Whittaker, Walkersville, Mass.), in six-well plates (BD Labware, Franklin Lakes, N. J.), at an initial concentration of 2×10^5 cells/ml. The plates were placed in a 5% CO_2-95% air fully humidified atmosphere at 37 °C and incubated until harvesting.

HL-60 cell differentiation along the granulocytic series was induced by a 5-day incubation with 1.25% of the polar-planer compound dimethyl sulfoxide (DMSO) or 100 nM retinoic acid (Sigma). Differentiation along the monocytic series was induced by a 5-day incubation with 100 nM 1,25-dihydroxyvitamin D_3 (Sigma). Differentiation toward macrophages was induced by a 3-day incubation with 1 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Steinheim, Germany).

Cellular surface and intracytoplasmic flow cytometric analysis

For the surface immunofluorescence analysis, cells were extracted from their culture media at the appropriate times, washed twice in PBS and stained at 4 °C for 15 min with the fluorescent-conjugated anti-CD10 or the corresponding fluorescent-labeled murine isotype-matched control antibody. The cells were then washed again in PBS and subjected to flow cytometric analysis on a FACScan (Becton Dickinson, Mountain View, Calif.) equipped with a 488-nM blue argon laser. Ten thousand events were recorded each time, and results were plotted on logarithmic scales. The percentage of cells expressing the antigen on their surface was calculated using Cell Quest software (Becton Dickinson).

For the intracytoplasmic analysis, cells were fixed and permeabilized using the two-step IntraStain from Dako, according to the procedure recommended by the manufacturer. The control antibody was always adjusted to the same concentration as the test antibody. The relative level of intracellular-detected antigen was quantified as mean fluorescence intensity. The changes in mean fluorescence intensity were evaluated after subtracting the background fluorescence observed in the cells stained with the corresponding FITC- or PE-conjugated isotype-matched control antibody.

Viability and apoptosis assays

Viability levels were assessed by determining the percentages of cells excluding the vital dye 7-AAD. Cells (5×10^5) were extracted from the culture medium, washed twice, then incubated for 20 min at room temperature with 5 µg/ml 7-AAD in PBS. After washing the cells again, the following flow cytometric analysis could discriminate between the cells which preserved their membrane integrity (viable cells) and cells which had lost their membrane integrity, having it permeabilized to the normal cell-impermeable dye.

3,3′-Dihexyloxacarbocyanine iodide is a mitochondrial transmembrane potentiometric fluorescent marker whose fluorescence decrease reveals the disruption of the mitochondrial transmembrane potential $\Delta\Psi m$, an early and irreversible step of ongoing apoptosis [19]. Cells (5×10^5) were incubated with 40 nM DiOC in PBS for 20 min at 37 °C, followed by flow cytometric analysis. In this assay, the apoptotic cells are identified by their decreased $\Delta\Psi m$ ($DiOC^{low}$).

The annexin V fluorescent assay was performed according to the manufacturer's instructions. Cells were extracted from the culture medium and washed twice with PBS; 5×10^5 cells were then stained with 2 µl annexin V FITC in the incubation buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$). To discriminate between early apoptotic cells and cells which have permeabilized their membranes (late apoptotic cells, or secondary necrotic), a counterstain with 5 µg/ml propidium iodide or 7-AAD was performed.

The Apo2.7 mAb reacts with a 38-kDa mitochondrial membrane protein (the 7A6 antigen) which is exposed on cells undergoing programmed cell death, its expression representing an early event of apoptosis rather than a final product of dead cells [20]. Cells (5×10^5) were fixed and permeabilized using the Dako IntraStain, following the manufacturer's instructions, washed and stained with 20 µl of Apo2.7 PE in 80 µl PBS, incubated for 15 min at room temperature, then washed again and subjected to flow cytometric analysis.

Poly (ADP-ribose) polymerase is a 116-kDa nuclear protein which plays a role in DNA repair and is strongly activated by DNA strand breaks, being cleaved during apoptosis by caspase-3 and -7 and yielding an 85-kDa and a 25-kDa fragment, this cleavage being considered one of the classical characteristics of apoptosis [21]. The anti-PARP cleavage site (214/215) antibody specifically recognizes the 85-kDa fragment of cleaved PARP. Cells (5×10^5) were fixed and permeabilized with Dako IntraStain, washed and stained with 10 ml of FITC-conjugated anti-PARP, incubated at 4 °C for 30 min, washed again and analyzed by flow cytometry.

Inhibitory assays

To test the effect of caspase inhibition on apoptosis features and NEP/CD10 expression, HL-60 cells were preincubated for 2 h with 25 µM z-vad.fmk. before exposure to jaspamide.

To inhibit protein synthesis during apoptosis induction by jaspamide, HL-60 cells were preincubated for 2 h with cycloheximide (50 µg/ml) before exposure to jaspamide.

To test the inhibition of the neutral endopeptidase activity, HL-60 cells were exposed to jaspamide in the presence of 1 µM phosphoramidon.

Statistical analysis

All experiments were performed in triplicate or quadruplicate. The paired or unpaired Student t test was used as appropriate to determine the statistical significance of the data obtained. The software used was StatView (Abacus Concepts, Berkeley, Calif.). A p value of < 0.05 was taken to represent a statistically significant difference between group means. Kolmogorov-Smirnov statistics were used to assess the flow cytometric histograms and the significance of their shifting.

Results

Jaspamide significantly decreases HL-60 cell viability by apoptosis induction

As shown in figure 1 A, jaspamide at concentrations of 100 nM and higher significantly decreased HL-60 cell vi-

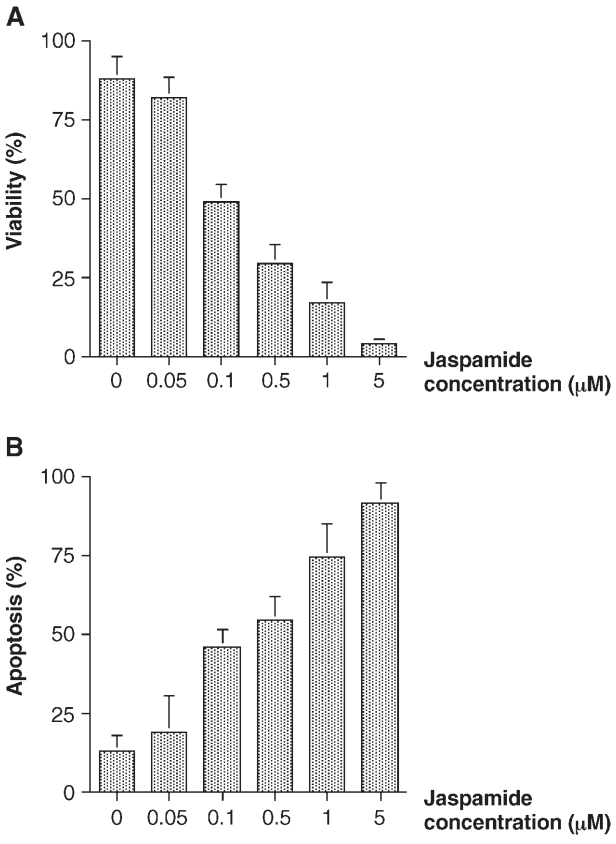

Figure 1. Jaspamide induces a significant decrease of viability by apoptosis induction in HL-60 cells. HL-60 cells were cultured in 10% fetal bovine serum RPMI 1640 medium for 48 h with different jaspamide concentrations (50 nM–5 μM), or without jaspamide (control samples), then extracted from their culture medium, washed and incubated for 20 min at room temperature with 5 μg/ml 7-AAD in PBS (*A*), or at 37 °C with 40 nm DiOC (*B*). The subsequent flow cytometric analysis quantified the percentages of cells which were 7-AAD positive or $DiOC^{low}$. Shown are the mean ± SD of four independent experiments.

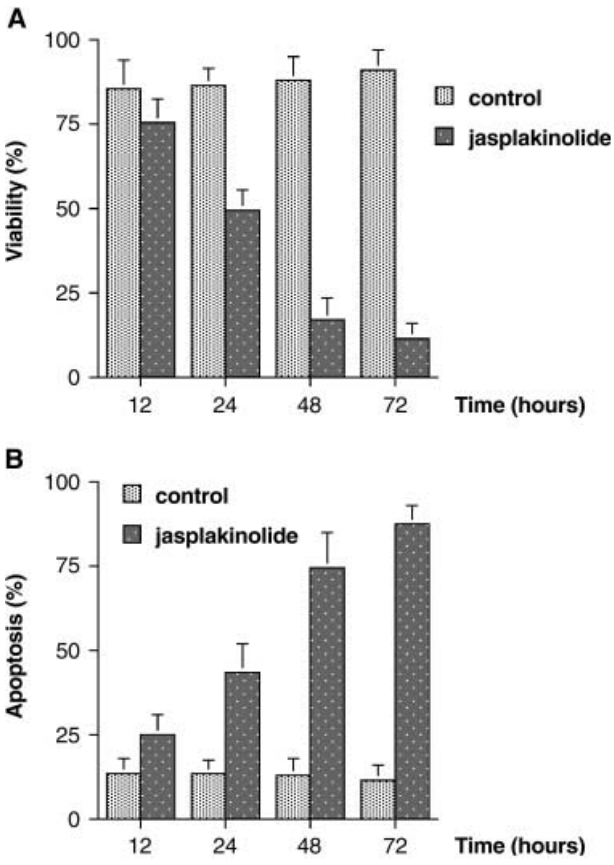

Figure 2. The time course of viability decrease and apoptosis induction in HL-60 cells incubated with jaspamide. HL-60 cells were cultured in 10% fetal bovine serum RPMI 1640 medium without jaspamide (control samples) or in the presence of 1 μM jaspamide. (*A*) The percentage of viable cells was determined by flow cytometry at various times thereafter, based on their ability to exclude 7-AAD dye. (*B*) The percentage of apoptotic cells was determined at various times, based on the decrease of mitochondrial transmembrane potential measured by DiOC fluorescence intensity. Shown are the mean ± SD of three independent experiments.

ability ($p < 0.01$, measured by their capacity to exclude cell-impermeable dyes like propidium iodide and 7-AAD) in a concentration-dependent manner. This significant reduction of survival was paralleled by a concentration-dependent increase of apoptosis (fig. 1B), the programmed cell death becoming statistically significant at similar jaspamide concentrations (100 nM and higher, $p < 0.01$). As shown in figure 2A, B, the viability decrease and apoptosis induction had already begun at 12 h and became significant at 24 h after incubation of HL-60 cells with 1 μM jaspamide.

The characteristics of the programmed cell death induction

As shown in figure 3A, apoptosis induction by jaspamide was quantified by four main methods, exploring the mitochondrial transmembrane potential decrease, phosphatidylserine externalization, PARP cleavage and expression of the mitochondrial 7A6 antigen, the apoptotic HL-60 cells displaying the nuclear (PARP), mitochondrial (DiOC and Apo2.7) and membrane (annexin V) features of programmed cell death induction.

The other important markers of programmed cell death induction are depicted in figure 3B. A significant increase in cells labeled with the anti-active caspase-3 antibody (which recognizes active caspase-3, as opposed to pro-caspase-3) demonstrated that the effector caspase cascade was activated by jaspamide (for 1 μM jaspamide 65 ± 11% vs 7 ± 3% control, $p < 0.05$).

The Bcl-2 oncoprotein is an integral inner mitochondrial membrane protein that blocks programmed cell death [22] and was found to be expressed in the myeloid lineage at the myeloblastic and promyelocytic stages of differentiation, including the human HL-60 cell line [23]. We found a significant decrease in Bcl-2 protein expression in HL-60 cells after jaspamide treatment (for 1 μM jas-

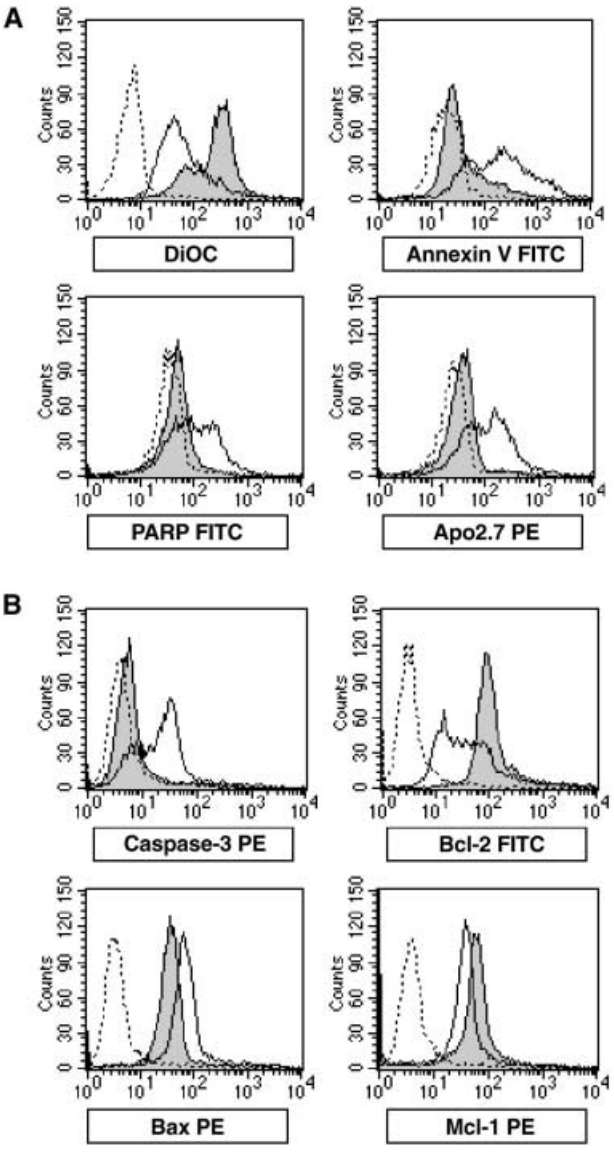

Figure 3. Characteristics of programmed cell death induced by jaspamide in HL-60 cells. Gray-filled histograms represent control cells (cultured without jaspamide) and solid-line empty curves represent cells incubated with jaspamide. The background fluorescence levels using unstained cells or a corresponding FITC- or PE-conjugated isotype-matched control mAb are also shown (dashed-line histograms). Representative of three independent experiments. (*A*) Incubation of HL-60 cells with 1 µM jaspamide for 48 h induced programmed cell death demonstrated by a decrease in the mitochondrial transmembrane potential $\Delta\Psi m$ (cells with decreased DiOC uptake), phosphatidylserine externalization (cells which became annexin V positive), PARP cleavage, and appearance of the apoptosis-associated 7A6 epitope (cells which became Apo2.7 positive). (*B*) The apoptosis-associated cellular events were caspase-3 activation (detected by a significant increase in the percentages of cells positive for active caspase-3), significantly decreased levels of Bcl-2 and Mcl-1 proteins (detected by a significant shift to the left of the cellular mean fluorescence intensities) and significantly increased Bax protein levels (evidenced by a significant shift to the right of the mean fluorescence intensity).

pamide MFI 59 ± 21 vs 198 ± 36 control, $p < 0.05$). We also found a significant increase in the pro-apoptotic Bax protein (for 1 µM jaspamide MFI 107 ± 28 vs 41 ± 16 control, $p < 0.05$), the heterodimerizing partner of Bcl-2, which determines apoptosis susceptibility and is designated as a death promoter [24]. The Bcl-2 family anti-apoptotic protein Mcl-1, which is localized predominantly in the endoplasmic reticulum and nuclear envelope [25], and which has similar functions as the Bcl-2 protein, was also found to be significantly decreased in HL-60 cells upon jaspamide treatment (for 1 µM jaspamide MFI 43 ± 17 vs 101 ± 24 control, $p < 0.05$).

The influence of caspase blocking on apoptosis induction by jaspamide

By using the nonselective, cell-permeable broad-range spectrum caspase inhibitor z-vad.fmk, we investigated programmed cell death induction following HL-60 cell incubation with jaspamide. As shown in figure 4, at both 100 nM and 1 µM jaspamide concentration, the nuclear features of apoptosis (PARP cleavage) were totally inhibited by 25 µM z-vad.fmk pretreatment, while the membrane (phosphatidylserine externalization) and mitochondrial (decrease of the transmembrane potential) features were not affected by the caspase inhibitor. These observations suggest that the jaspamide-induced PARP cleavage is dependent on activation of caspases, while the disruption of the mitochondrial potential and the phosphatidylserine externalization are not.

CD10 expression following incubation with jaspamide

Following a 48-h incubation with jaspamide concentrations of 100 nM and higher, neutral endopeptidase/CD10 expression was detected on the surface of apoptotic HL-60 cells, as shown in figure 5. This NEP/CD10 appearance was detected by two different mAbs (SS2/36 and ALB1), and the jaspamide-treated HL-60 cells did not fix two control antibodies (anti-CD5 and anti-CD19), demonstrating the specificity of anti-CD10 mAbs.

CD10 was not intracytoplasmically presynthesized, since permeabilized control cells failed to demonstrate CD10 intracellular expression. When HL-60 cells were preincubated for 2 h with 50 µg/ml cycloheximide before a 48-h jaspamide incubation, CD10 did not appear, indicating that CD10 was synthesized de novo by the HL-60 cells (data not shown).

When HL-60 cells were induced to differentiate along the granulocytic series by a 5-day preincubation with 100 nM retinoic acid or 1.25% DMSO, apoptosis was induced (retinoic acid: 26 ± 6% control vs 60 ± 8% jaspamide, $p < 0.05$; DMSO: 19 ± 5% control vs 57 ± 7% jaspamide, $p < 0.05$) and CD10 expression appeared on the surface of the differentiated HL-60 cells after a 48-h incubation with 0.5 µM jaspamide. However, when HL-60 cells were

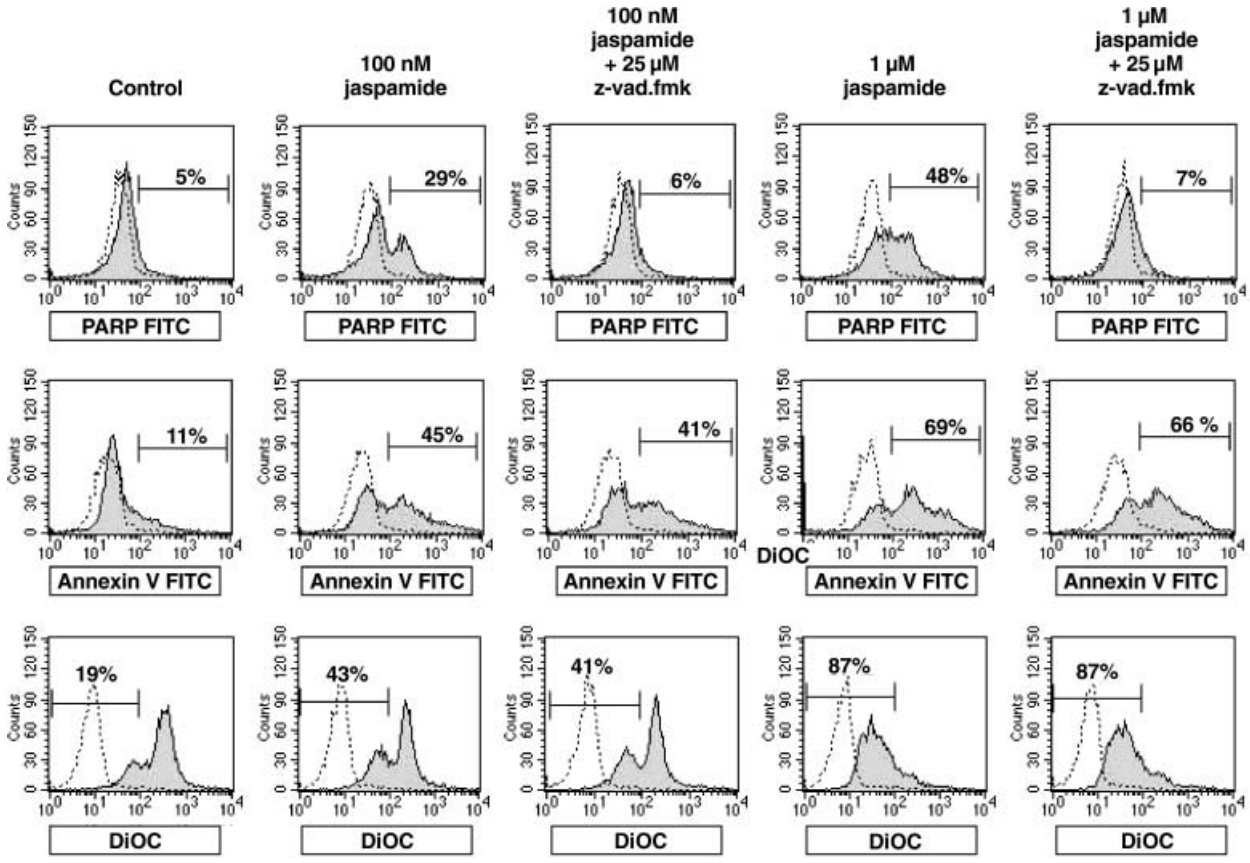

Figure 4. The effects of the broad-range caspase inhibitor z-vad.fmk on apoptosis induced by jaspamide. After 2 h preincubation with 25 µM of the caspase inhibitor z-vad.fmk, HL-60 cells were incubated for 48 h in the absence of jaspamide (control cells) or in the presence of 100 nM and 1 µM jaspamide. At 48 h, the nuclear (PARP cleavage), mitochondrial (DiOC uptake) and membrane (phosphatidylserine externalization) features of apoptosis were examined. The percentages of apoptotic cells as determined by the three methods are depicted in each histogram. Dashed lines represent the background staining determined by a FITC-conjugated isotype-matched control mAb or using unstained cells. Representative for three separate experiments.

induced to differentiate along the monocytic series by a 5-day incubation with 100 nM 1,25-dihydroxyvitamin D_3 or toward macrophages by a 3-day preincubation with 1 ng/ml PMA, CD10 failed to appear on the cell surface after 48 h incubation with 0.5 µM jaspamide (fig. 6), even if apoptosis was induced (vitamin D_3: 16 ± 5% control vs 58 ± 7% jaspamide, $p < 0.05$, PMA: 11 ± 4% control vs 51 ± 6% jaspamide, $p < 0.05$). Furthermore, the U937 promonocytic cell line and the THP-1 mature monocytic cell line were induced into apoptosis, but failed to express NEP/CD10 after 48 h incubation with 0.5 µM jaspamide.

The newly synthesized NEP/CD10 interferes with jaspamide apoptosis induction

To test the hypothesis that neutral endopeptidase interferes with jaspamide activity by inhibiting its effect on HL-60 cells, the neutral endopeptidase inhibitor phosphoramidon or saturating concentrations of anti-CD10 mAbs were used to block the neutral endopeptidase activity. As shown in figure 7, neither phosphoramidon nor anti-CD10 mAbs induced apoptosis by themselves, but when HL-60 cells were incubated with jaspamide in the presence of phosphoramidon or anti-CD10 mAbs, apoptosis induction was significantly enhanced (control 12 ± 5% apoptotic cells, 0.5 µM jaspamide 51 ± 7% apoptotic cells, 0.5 µM jaspamide plus 50 µl/ml anti-CD10 mAb 73 ± 8% apoptotic cells, $p < 0.05$; 0.5 µM jaspamide plus 1 µM phosphoramidon 88 ± 7% apoptotic cells, $p < 0.05$).

A connection between apoptosis induction and NEP/CD10 expression

As shown in figure 8, using two-color flow cytometric analysis, the cells which were commited to programmed cell death were demonstrated to express CD10 on their surface upon incubation with jaspamide. The cells expressing the CD10 molecule on their surface were the cells which had a decreased mitochondrial transmembrane potential and were committed to programmed cell death [19].

To test the hypothesis that caspase inhibition blocks the appearance of CD10 on the surface of jaspamide-treated cells, the z-vad.fmk broad-range caspase inhibitor was used at 25 µM, concentration which completely blocked

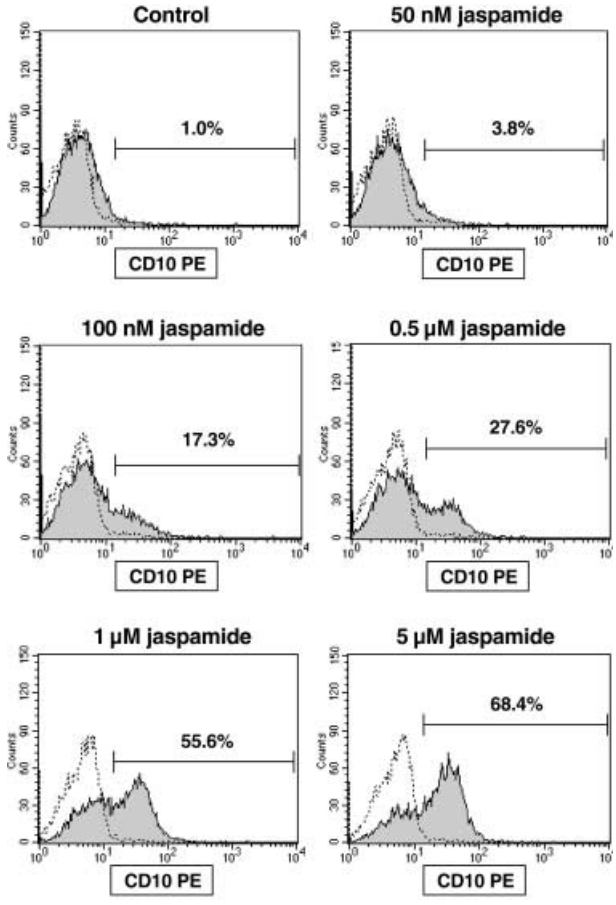

Figure 5. CD10 appearance on the surface of HL-60 cells incubated with jaspamide. HL-60 cells were incubated for 48 h with jaspamide concentrations ranging from 50 nM to 5 µM, or without jaspamide (control cells). At 48 h cells were harvested, stained with 20 µl PE-conjugated anti-CD10 mAb and analyzed by flow cytometry using the FL2 detector for surface CD10 expression. The percentages of CD10-positive cells are depicted in each histogram. The dotted lines represent the background staining as determined by a PE-conjugated isotype-matched mAb. The results shown are representative of four independent experiments.

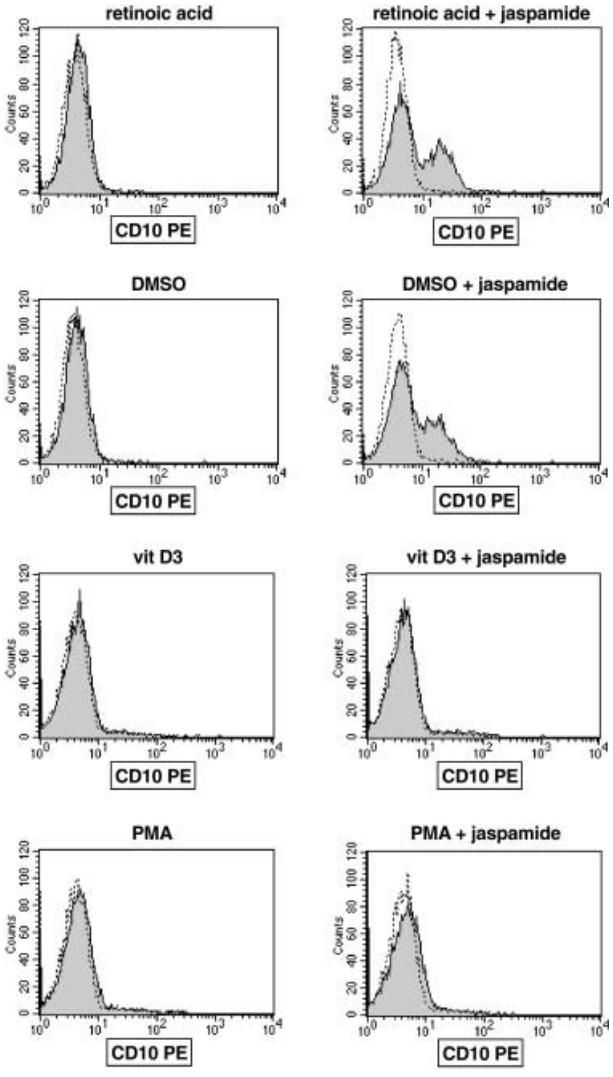

Figure 6. The effects of differentiating agents on CD10 expression. HL-60 cells were differentiated along the granulocytic series by a 5-day preincubation with 100 nM retinoic acid (*A*) and 1.25% DMSO (*B*), or along the monocytic series by a 5-day preincubation with 100 nM 1,25-dihydroxyvitamin D_3 (*C*) or along the macrophage series by a 3-day preincubation with 1 ng/ml PMA (*D*). Following a 48-h incubation with 0.5 µM jaspamide, the cells were harvested, stained with PE-conjugated anti-CD10 mAb and analyzed by flow cytometry using the FL2 detector for surface CD10 expression. The dotted lines represent the level of fluorescent background determined by staining with a PE-conjugated isotype-matched mAb. The results shown are representative.

PARP cleavage (fig. 4) and caspase-3 activation (control 7 ± 3% positive cells, 1 µM jaspamide 65 ± 11% positive cells, 1 µM jaspamide plus 25 µM z-vad.fmk 2 ± 1% positive cells, $p < 0.05$), programmed cell death induction being inhibited and the cells remaining viable (control 89 ± 7% 7-AAD-negative cells,1 µM jaspamide 19 ± 6% 7-AAD-negative cells, 1 µM jaspamide plus 25 µM z-vad.fmk 91 ± 6% 7-AAD-negative cells, $p < 0.05$). As shown in figure 9, at this z-vad.fmk concentration, CD10 appearance was partially, but not totally inhibited, at both 100 nM and 1 µM concentrations of jaspamide.

Discussion

This study showed that HL-60 cells cultured in the presence of jaspamide at concentrations of 100 nM and higher undergo programmed cell death, and also that, at the same concentrations, CD10/neutral endopeptidase is expresed on the surface of these cells committed to apoptosis. First, the jaspamide concentrations at which spontaneous apoptosis is significantly increased (100 nM) are in agreement with our previous observations [7] that

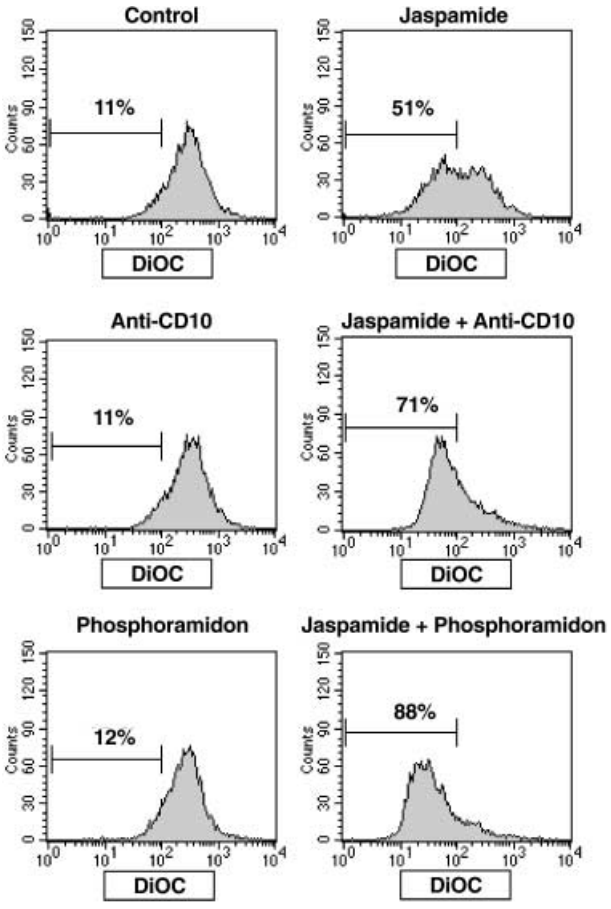

Figure 7. Inhibition of CD10/neutral endopeptidase enhances apoptosis induction by jaspamide in HL-60 cells. HL-60 cells were cultured for 48 h without any reagent (control), or in the presence of 50 µl/ml anti-CD10 mAb, or 1 µM phosphoramidon, or 0.5 µM jaspamide, or 0.5 µM jaspamide plus 50 µl/ml anti-CD10 mAb, or 0.5 µM jaspamide plus 1 µM phosphoramidon. Percentages of apoptotic cells were then evaluated by flow cytometry using the FL1 detector to assess DiOC fluorescence intensity as an indicator of mitochondrial transmembrane potential. The results shown are representative for three independent experiments.

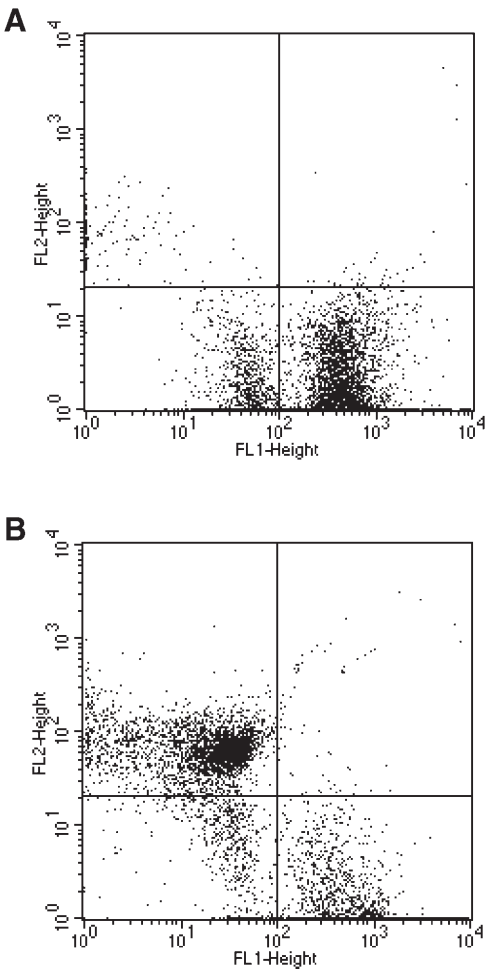

Figure 8. HL-60 cells expressing surface CD10 after incubation with jaspamide are committed to the programmed cell death pathway. HL-60 cells were cultured for 48 h in the presence of 1 µM jaspamide or without jaspamide (control cells), harvested and stained simultaneously with the mitochondrial-potential marker DiOC and with a PE-conjugated anti-CD10 mAb, and then subjected to two-color flow cytometric analysis. FL1 detector was used to detect the DiOC fluorescence, and FL2 was used for the PE-conjugated anti-CD10 mAb. Control HL-60 cells do not display CD10, either on the surface of viable cells nor on the apoptotic cell surface (*A*). HL-60 cells committed to programmed cell death after incubation with jaspamide displayed CD10 (*B*). The results shown are representative for four separate experiments.

10 nM of jaspamide had little effect, while 50 nM jaspamide demonstrated an inhibitory effect on cell proliferation, these results being concordant with other studies which reported a 50% inhibitory concentration of 35 nM on PC3 prostate carcinoma cells [26], or 41 nM for LNCaP, 65 nM for PC3 and 179 nM for TSU-Pr1 cell lines [5], or 35 nM for the murine cell line CTLL-20 [27]. In this last study on murine CTLL-20 cells, the authors reported that concentrations of 25 nM and below had no effect on the apoptotic process, whereas concentrations as low as 50 nM were sufficient to enhance apoptosis; the percentage of apoptotic cells at the 100 nM jaspamide concentration (56%) was close to our results (45.8%) at the same jaspamide concentration.

Regarding the apoptosis-associated markers, enhanced expression of caspase-3 and decreased Bcl-2 protein were demonstrated to be associated with apoptosis induction in HL-60 cells [28], and also decreased levels of Mcl-1 protein, which has a function analogous to Bcl-2 in HL-60 cells [29]. Our findings demonstrate that jaspamide-induced apoptosis is also associated with caspase-3 activation and a decrease in Bcl-2 and Mcl-1 protein expression, but also with increased Bax levels. Bax is responsible for a caspase-independent cell death pathway [30], which could explain our findings that even if caspases were blocked by the broad-range caspase inhibitor z-vad.fmk, the mitochondrial potential disruption and membrane phosphatidylserine externalization still ap-

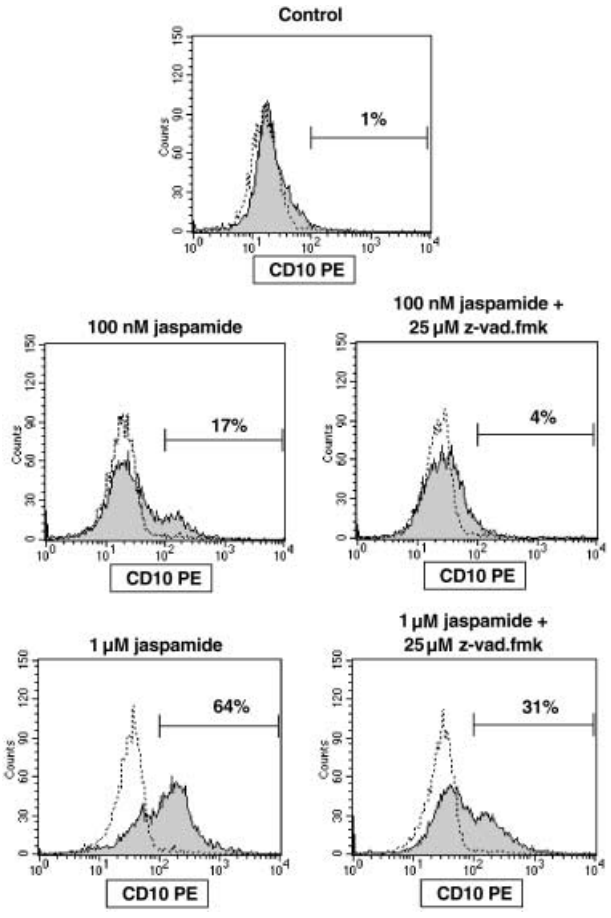

Figure 9. CD10 expression on the surface of HL-60 cells is partially, but not totally inhibited by preincubation with z-vad.fmk. HL-60 cells (5×10^5) were cultured for 48 h without jaspamide (control cells), or in the presence of 100 nM or 1 µM jaspamide, with or without 2 h preincubation with 25 µM of the broad-range caspase inhibitor z-vad.fmk, then harvested, stained with 20 µl of PE-conjugated anti-CD10 and analyzed by flow cytometry using the FL2 detector to evaluate the CD10 surface expression. Gray-filled histograms represent CD10 expression, and dotted lines are the level of fluorescent background obtained with a PE-conjugated isotype-matched control mAb. Results shown are representative for four independent experiments.

peared. Thus, it seems that jaspamide induces a caspase-independent pathway of cell death, which is responsible for the observed cytoplasmic and membrane changes in apoptosing cells, and also a caspase-dependent cell death, which is responsible for PARP proteolysis. This is in agreement with other observations that caspases influence the mode but not the extent of cell death, caspase activity being required for PARP cleavage and DNA fragmentation, other manifestations of cell death (mitochondrial depolarization, exposure of phosphatidylserine, cell membrane disruption and cell shrinkage) being caspase independent [31].

The second important finding in this study is the CD10/neutral endopeptidase expression on the surface of the apoptotic HL-60 cells. Like human T cells, which do not express CD10 on their surface, but are induced to synthesize it upon apoptosis induction by anti-CD95 mAb, etoposide or staurosporine treatment [14], we found that HL-60 cells, which do not normally express CD10 either on their surface [10, 15] or intracytoplasmically, can be induced to synthesize and express surface CD10 after jasplakinolide treatment.

Because mature granulocytes express CD10 [10], and because jasplakinolide is able to induce immunophenotypic maturation of HL-60 cells, with upregulation of CD14 and CD11 and downregulation of CD34 antigens [32], with upregulation of CD16 and CD14B and with a significant increase in the percentage of cells exhibiting polylobulated nuclei [33] or with a significant increase in the percentage of polynuclear cells [7], CD10 expression on HL-60 cells incubated with jaspamide could be regarded as related to granulocytic differentiation. Relevant in this respect are the findings that HL-60 cells differentiated with retinoic acid and DMSO (along the granulocytic series) maintained their CD10-expressing capability, while HL-60 cells differentiated with vitamin D_3 and PMA (toward monocytes and macrophages, respectively) lost their CD10-expressing capability. Furthermore, the monocytic-lineage-committed cell lines U937 and THP-1 failed to express CD10 upon incubation with similar jaspamide concentrations. The physiological implication of these observations is that CD10 expression on the granulocytic surface could be considered a marker of increased pro-apoptotic potential. Indeed, HL-60 cells differentiated toward the granulocytic lineage suffer a significant reduction in Bcl-2 expression [23] and acquire an increased propensity to undergo programmed cell death compared with nondifferentiated cells [34].

The biologic activity of NEP/CD10 in renal proximal tubules and the small intestine is to participate in the final stages of peptidic bond hydrolysis, with a preference for small peptides, on the NH_2 terminal side of hydrophobic amino acids [10, 11]. Jaspamide has a small and hydrophobic molecule, containing three hydrophobic amino acid residues (alanine, tryptophan and tyrosine) [26], which makes it a perfect target for NEP/CD10. Thus, CD10/NEP is synthesized de novo in HL-60 cells incubated with jaspamide, and tends to decrease the jaspamide effect on HL-60 cells, probably by hydrolyzing one of the peptidic bonds of the jaspamide molecule, reducing in this way the concentration of the active peptide surrounding the cells. Thus, jaspamide could be regarded as an immunomodulator directed against its triggering signal, creating a local cellular negative regulatory loop. This would explain our findings of apoptosis augmentation by CD10/NEP inhibition with phosphoramidon or anti-CD10 mAbs. This finding of amplification of target peptide activity by blocking CD10/NEP is similar to results obtained for other biological peptides which are hydrolyzed by NEP/CD10: inhibition of NEP/CD10 en-

zymatic activity reduced the amount of met-enkephalin required to trigger shape and size changes in human neutrophils [35], potentiates the bacterial chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine and substance P actions on human neutrophils [36] and augmented all the effects of atrial natriuretic peptide and brain natriuretic peptide on neutrophil functions [37].

Our results define a connection between the two observed jaspamide effects on HL-60 cells: programmed cell death induction and CD10 expression. The fact that CD10 was expressed by the cells committed to the programmed cell death pathway, and that by blocking caspase activation with the broad-range caspase inhibitor z-vad.fmk, CD10 expression was significantly decreased, strongly suggest that a connection exists between the two processes. The reason why CD10 expression was partially, but not totally inhibited may be related to the observed caspase-independent component of the programmed cell death induced by jaspamide in HL-60 cells.

The findings presented in this work show that HL-60 promyelocytic leukemia cells are induced to undergo programed cell death by incubation with jaspamide, and that this apoptosis induction was accompanied by CD10/neutral endopeptidase expression on the surface of the apoptotic cells. To our knowledge, we are the first to describe the caspase-dependent and -independent pathway of programmed cell death induced by jasplakinolide and also the first to point to the association between apoptosis induction and CD10/neutral endopeptidase expression in a myeloid cell line. Finally, based on the results presented in this study, further research is mandated to evaluate a possible therapeutical approach using jasplakinolide in human acute myeloid leukemia.

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