Cell Density-dependent Apoptosis in HL-60 Cells, Which Is Mediated by an Unknown Soluble Factor, Is Inhibited By Transforming Growth Factor β1 and Overexpression of Bcl-2

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Kumiko Saeki‡, Akira Yuo‡§, Mitsuyasu Kato‡, Kohei Miyazono‡, Yoshio Yazaki¶ and Fumimaro Takaku‡

From the ‡Department of Hematology, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162, the §Department of Biochemistry, Cancer Institute, Tokyo 170, and ¶The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113, Japan

We report a novel mode of apoptosis induction observed in human leukemic HL-60 cells. These cells spontaneously underwent apoptosis in the course of proliferation when the cell density became higher than $1 \times 10^7$/ml. This occurred under ordinary in vitro culture conditions, with or without fetal calf serum. Even the low density cells were committed to undergo apoptosis if they were cultured under artificially concentrated conditions. Replacement of the culture supernatant of the low density cells by that of the high density ones resulted in apoptosis induction in the former cells. This apoptosis-inducing activity of the high density cell culture supernatant was completely eliminated by the action of trypsin but was fully restored following ultrafiltration by 3-kDa pore-sized membrane. A strong apoptosis-inducing activity was recovered from the culture supernatant of the high density HL-60 cells at a specific fraction in reverse-phase column chromatography. Neither an interleukin-β converting enzyme inhibitor nor CPP-32 inhibitor blocked the induction of cell density-dependent apoptosis in HL-60 cells, although overexpression of Bcl-2 protein markedly attenuated the induction of this mode. Surprisingly, transforming growth factor-β1 and activin A did not induce but, rather, inhibited the induction of cell density-dependent apoptosis. These data suggest that HL-60 cells release an unknown low molecular weight peptide-containing factor in response to an increase in cell density to induce apoptosis in an autocrine manner and that the interleukin-β converting enzyme-independent intracellular machinery for this mode of apoptosis is strongly affected by signaling events through the transforming growth factor-β receptor and by the action of Bcl-2 oncoprotein.

Apoptosis, or programmed cell death, is an important biological process that is indispensable in normal development or differentiation, preservation of homeostasis, and liberation of hosts from viral attacks or spread of malignant cells (1–3). Apoptosis is observed under in vitro culture conditions as well as in vivo. According to previous reports on its induction in cultured cell lines, the modes of induction in vitro fall into three categories. First, a wide variety of noxious stimulations can bring on the condition. For example, addition of the anticancer drugs actinomycin D (4) or etoposide (5) and cytotoxic cytokines such as tumor necrosis factor α (TNF-α) (6) or transforming growth factor β1 (TGF-β1) (7) to the culture media of hematopoietic cells results in apoptosis. The high osmotic pressure of the culture medium also induces apoptosis in epithelial cell lines (8). In addition, heat shock stimulation (9) and ultraviolet (10) or gamma ray irradiation (11) induce apoptosis in some cases.

Second, the absence of cytoprotective signals results in apoptosis in particular cases. For example, rat pheochromocytoma-derived PC-12 cells undergo apoptosis after the deprivation of nerve growth factor from the culture medium (12), and the murine pro-B cell derived Ba/F3 cells do so after the depriva-
tion of interleukin-3 (13).

Finally, apoptosis is occasionally observed following the differentia-
tion of cells. For example, all-trans-retinoic acid-treated human leukemic HL-60 cells are reported to undergo apoptosis after having been differentiated to granulocytic lineage (14).

In all these situations, however, certain positive or negative environmental changes are required to induce apoptosis. It is not yet clear whether exogenous forces which change the cellular environment are always required, and to address this question, we investigated whether the increase of cell density, which is an internal stress, could possibly trigger apoptosis. We cultured human leukemic HL-60 cells under ordinary in vitro culture conditions and examined apoptosis induction over a period of time.

In this paper, we show that these cells spontaneously undergo apoptosis in the course of their proliferation without any external stress. We also show evidence to prove that this spontaneous apoptosis is triggered by an increase in cell density per se, which is a novel mode of induction. Finally, we show that the cell density-dependent apoptosis is mediated by an unknown soluble factor, present in the conditioned medium of these cells themselves. Our work describes a novel mechanism of cell auto-regulation: self-induced apoptosis in an inappropriately crowded condition.

MATERIALS AND METHODS

Cells, Cytokines, and Inhibitors—HL-60 cells were maintained in RPMI 1640 medium (Life Technologies Inc.) supplemented with 10% heat-inactivated fetal calf serum (JRH Bioscience, Lenexa, KS). Cells

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† To whom correspondence should be addressed: Dept. of Hematol-
ogy, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162, Japan. Tel.: 81-3-3202-7181 (ext. 2807); Fax: 81-3-3207-1038.

1 The abbreviations used are: TNF-α, tumor necrosis factor α; TGF-
β1, transforming growth factor β1; HP, hematoregulatory peptides; ICE, interleukin-1β converting enzyme; R, receptor.
were passaged every 2 days at an initial concentration of 6.5 x 10^6/ml. A bcl-2 overexpressing derivative line of HL-60 was the kind gift of Prof. S. Kitagawa and Dr. Y. Furukawa (15), and was maintained in 200 µg/ml genetin (Sigma). Recombinant human TGF-β1 and activin A were generously provided by Kirein Brewery Co., Ltd. (Tokyo, Japan) and Allolimoto Company Inc. (Kawasaki, Japan), respectively. Recombinant human TNF-α was purchased from Pepro Tech EC, Ltd. (London, UK). The interleukin-1β converting enzyme (ICE) inhibitor II (Bachem Feinchemikalien AG, Bubendorf, Switzerland) and CPP-32 inhibitor (Peptide Institute Inc., Osaka, Japan) were dissolved in MeSO and kept at ~20°C until use.

Trypsin Treatment of Conditioned Medium—Trypsin from bovine pancreas (Sigma) was suspended in 50 mM Tris at pH 8.0 (5% Triton X-100, 10 mM EDTA), then centrifuged at 15,000 rpm at room temperature for 20 min, and the supernatant was treated with 1 mg/ml heat-inactivated RNase A (Sigma) at 45°C for 90 min and with 200 µg/ml proteinase K (Roehringer Mannheim, Mannheim, Germany) for another 60 min. After phenol extraction, phenol/chloroform extraction, and ethanol precipitation, one-third of the DNA sample was applied on 2% agarose gel, separated by horizontal electrophoresis, and visualized by staining with ethidium bromide (500 ng/ml). Marker 4 (WAKO Chemicals Co., Ltd., Osaka, Japan) was used for the evaluation of the molecular weights of the fragmented DNA.

In some experiments, cytoplasmic DNA was used to evaluate apoptosis. 3 x 10^6 cells were lysed with 350 µl of lysis buffer containing 0.6% SDS and 0.1% EDTA (pH 8.0), 0.5% Triton X-100, 10 mM EDTA, then centrifuged at 15,000 rpm at room temperature for 20 min, and the supernatant was treated with RNase A and proteinase K as described above. The cells were washed with phosphate-buffered saline and fixed on slide glasses using a cytospin apparatus (Cytospin2, Shandon, Pittsburgh, PA). After being dried in air and fixed with a mixture of acetone/methanol (3:1) for 5 min at room temperature, the samples were incubated with 4 µg/ml H33342 (Calbiochem) for 10 min at room temperature. The chromatin structures of the cells were examined by fluorescence light microscopy.

Preparation of Conditioned Medium—HL-60 cells were seeded at an initial density of 1 x 10^5/ml in RPMI 1640 medium supplemented with 10% fetal calf serum. After 24 h of incubation at 37°C, the cells were washed and resuspended with serum-free RPMI 1640 medium supplemented with 5 µg/ml bovine pancreas insulin (Sigma) and 5 µg/ml human holo-transferrin (Sigma). Following another 72 h incubation at 37°C, the supernatant was collected and stored at ~80°C until use.

Trypsin Treatment of Conditioned Medium—Type III trypsin from bovine pancreas (Sigma) was suspended in 50 mM Tris at pH 8.8 (i.e. 5% trypsin solution). We added 120 µl of this solution to 3 ml of conditioned medium of HL-60 cells, incubated it for 30 min at 37°C, and then used it for the assay. For incubation of trypsin, this was ultrafiltered with a 3-kDa pore-sized membrane (Millipore Co., Bedford, MA) or boiled at 100°C for 5 min.

Column Chromatography—Twenty-five-ml of the conditioned medium of HL-60 cells was diluted four times with 10 mM Tris (pH 7.5). This diluted conditioned medium was then applied to a 20-ml column (Econopack column, Bio-Rad) packed with 5 ml of CM-Sepharose (Sigma), which had previously been equilibrated with 10 mM Tris (pH 7.5). After the column was washed with 15 ml of 10 mM Tris (pH 7.5), the sample was eluted with 10 ml of elution buffer (10 mM Tris (pH 7.5) with 150 mM NaCl). Sodium elution buffer was then applied to a reverse-phase column (ProRPC, Pharmacia Biotech, Uppsala, Sweden). The elution was performed using a gradient mixture of water and acetonitrile, both of which were supplemented with 0.1% trifluoroacetic acid. The elution pattern was monitored by checking the absorbance value of the ultraviolet wave (at a wavelength of 215 nm).

RESULTS

HL-60 Cells Spontaneously Underwent Apoptosis during the Course of Proliferation in Vitro—There appeared an increasing number of dead cells among the in vitro cultured HL-60 cells with the passage of time. In the initial experiment, we performed DNA fragmentation assay and morphological examination to determine whether they died by apoptosis.

Fig. 1A is the growth curve of HL-60 cells. They exponentially grew as long as their density was less than 8 x 10^5/ml, and no sign of apoptosis was detected in the DNA fragmentation assay (Fig. 1B, lanes 1 and 2) or the morphological examination (Fig. 1C, upper). However, apoptotic cells appeared when the cell density exceeded 1 x 10^5/ml (Fig. 1C, lower), in which internucleosomal DNA fragmentation was actually detected (Fig. 1B, lanes 3 and 4). Similar results were obtained when HL-60 cells were cultured in a serum-free condition and supplemented with insulin and transferrin (data not shown). These results indicate that HL-60 cells undergo apoptosis without known apoptotic stimuli during in vitro culture.

HL-60 Cells Were Committed to Undergoing Apoptosis When Cultured in an Artificially Concentrated Condition—To identify the mechanisms of spontaneous apoptosis in HL-60 cells, we investigated whether the increase of cell density per se could possibly be a trigger for the apoptosis induction. The culture supernatant of the growing phased cells (4 x 10^5/ml) was partially removed so that the cells were in a 4 fold-concentrated condition (i.e. 1.6 x 10^6/ml). After 30 min incubation at 37°C they were diluted with fresh medium to the original concentration (i.e. 4 x 10^5/ml), and after another 10 h incubation, DNA fragmentation assay was performed. For controls, cells that were cultured without having been concentrated were used. As shown in Fig. 2, the cells that had been cultured in a
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Culture Supernatant of High Density HL-60 Cells Had the Ability to Induce Apoptosis in Low Density Cells—To learn whether the cell density-dependent apoptosis in HL-60 cells was mediated by a soluble factor, the culture supernatant of low density cells ($3.5 \times 10^6/ml$) was replaced by that of high density cells ($1.6 \times 10^6/ml$) in various proportions. After 10 h incubation at 37 °C, DNA fragmentation assay was performed. As shown in Fig. 3A, addition of the high density cell culture supernatant induced DNA fragmentation in the low density cells in a dose-dependent manner. Similar results were obtained when this supernatant was prepared from cells maintained in a serum-free condition (data not shown). These results indicate that the high density cells culture supernatant has an ability to induce apoptosis in low density cells.

To determine whether the culture supernatant of the artificially concentrated HL-60 cells also had an apoptosis inducing activity, the growing phased cells ($6 \times 10^5/ml$) were incubated in a 3-fold concentrated condition ($1.8 \times 10^5/ml$) by removing two-thirds of the culture supernatant. After incubation for 30 min or 6 h at 37 °C, the culture supernatants of these artificially concentrated cells were collected, and their apoptosis inducibility was assayed. The supernatant of the same growing phased cells that had been cultured without concentration was used as control. As shown in Fig. 3B, addition of the artificially concentrated cells' culture supernatant caused apoptosis in the growing phased cells, suggesting that the artificially concentrated HL-60 cells release an inducer of apoptosis into the medium within a short period of cultivation in response to the high cell concentration.

Characterization and Partial Purification of a Soluble Apoptosis-inducing Factor from HL-60 Cells—To characterize the soluble apoptosis-inducing activity in the conditioned medium of HL-60 cells, we studied the effect of trypsin treatment of the high density cell culture supernatant on the induction. The conditioned medium, which was prepared from HL-60 cells cultured in a serum-free condition, was treated with trypsin or buffer only, and the apoptosis-inducing activity was assayed. As shown in Fig. 4, the trypsin-treated culture supernatant did not induce apoptosis at all in the exponentially growing phased cells. This finding indicates that the cell density-dependent apoptosis in HL-60 cells is mediated by a soluble factor that is sensitive to trypsin treatment. In examining the chemical or mechanical stability of this apoptosis-inducing activity, we found that it was completely resistant to mild (56 °C, 30 min) and severe heat treatment (100 °C, 5 min) and also to transient alkaline (pH 11) or acid (pH 3.5) treatment (data not shown).

To further confirm the existence of certain apoptosis-inducing molecules in the culture supernatant, we partially purified this factor. The HL-60 cell conditioned medium was first semi-purified by anion exchange column chromatography. Twenty-five ml of the medium was diluted four times with salt-free buffer and applied to CM-Sepharose column, and stepwise elution was performed using buffer containing 150 mM, 500 mM, or 1000 mM NaCl. Because we found that the apoptosis-inducing activity was concentrated in the 150 mM NaCl-eluted fraction (data not shown), we applied this fraction to reverse-phased column chromatography, and elution was performed.
without TGF-β showed apparent growth advantage as compared with those exerting any effect on the proliferation of exponential growth shown. We also found that TGF-β did not induce apoptosis by itself and neither enhanced nor suppressed induction in human leukemic U937 cells (6), did not induce but, rather, strongly inhibited cell density-dependent apoptosis in HL-60 cells (data not shown). The anti-apoptotic effect of TGF-β may be due either to HL-60 cells' decreased production of apoptosis-inducing factor or their acquisition of resistance to it. To address this question, HL-60 cells were cultured with or without TGF-β, and the culture supernatant was prepared, ultrafiltrated through 3-kDa pore-sized membrane to deplete any residual TGF-β, and added to the exponentially growing cells to evaluate the apoptosis-inducing activity. As shown in Fig. 6F, coexistence of TGF-β did not reduce the apoptosis inducibility of the conditioned medium, suggesting that TGF-β-treated cells still release apoptosis inducer into the culture medium without being compelled to undergo apoptosis themselves. We also examined the effect of TGF-β1 on the apoptosis induced by exogenously added soluble inducer. As shown in Fig. 6G, TGF-β1 almost completely abrogated DNA ladder formation in HL-60 cells cultured with conditioned media of high cell density culture. These findings clearly indicate that TGF-β1 inhibits cell density-dependent apoptosis not by simple reduction of the release of soluble apoptosis inducer but, rather, by interaction of its intracellular signaling pathways with those activated by high cell density and/or soluble inducer.

Overexpression of Bcl-2 Protein Strongly Attenuated Induction of Cell Density-dependent Apoptosis but Did Not Inhibit Release of an Apoptosis Inducer—To explore whether the overexpression of Bcl-2 protein could inhibit the induction of cell density-dependent apoptosis in HL-60 cells, two derivative lines of these cells, in which the expression vector of bcl-2 gene or neomycin-resistant gene was stably transfected (15), were cultured, and the DNA fragmentation assay was performed in accordance with the time course. Overexpression of Bcl-2 protein did not affect the growth rate of the cells (Fig. 7A); however, it markedly attenuated the induction of cell density-dependent apoptosis (Fig. 7B).

The resistance to cell density-dependent apoptosis in Bcl-2 overexpressing HL-60 cells was due either to the decreased production of apoptosis-inducing factor or the decreased sensitivity to this factor. To address this question, conditioned media of Bcl-2 overexpressing and the control neomycin-resistant HL-60 cells were added to the exponentially growing parental HL-60 cells, and DNA fragmentation assays were performed as before. As shown in Fig. 7C, the conditioned media of both cell lines showed similar apoptosis-inducing activities, suggesting that the overexpression of Bcl-2 protein did not inhibit production of the inducer but blocked the intracellular signaling events that lead to the induction.
apoptosis in HL-60 cells may be mediated by ICE/CPP-32 independent pathways.

**DISCUSSION**

We demonstrated that human myeloblastic HL-60 cells underwent apoptosis strictly dependent on the cell density during *in vitro* culture with or without fetal calf serum. The cell density-dependent apoptosis was, at least in part, mediated by a soluble factor, and the autocrine or paracrine mediator was found to be a low molecular weight peptide-containing molecule. In regard to the intracellular mechanisms of this novel mode of apoptosis, ICE-related pathways, ICE by itself and its downstream molecule of CPP-32, are not likely to be involved, but the Bcl-2-sensitive component would contribute to the intracellular signaling of cell density-dependent apoptosis. Interestingly, TGF-β family cytokines potently inhibited this novel mode of apoptosis in HL-60 cells.

As shown, this apoptosis-inducing factor seems to be a small peptide whose molecular mass is less than 3 kDa. Our preliminary observation shows that the factor is even smaller than 1 kDa, which indicates that it may be composed of only 5 to 10 amino acids. There are various kinds of physiologically active oligopeptides, including hormones and neurotransmitters. Among these substances, a pentapeptide called hematoregulatory peptide (HP), which was isolated from the conditioned media of mature granulocytes as an inhibitor against the formation of myeloipoietic colonies, was reported to inhibit the growth of HL-60 cells (reviewed by Paukovits *et al.* (17)). We do not know whether HP can also induce apoptosis in HL-60 cells. However, it is not likely that this peptide would be the factor we are trying to isolate, because HP is reported to be completely resistant to the action of trypsin.

Are any cytotoxic cytokines involved in the induction of the cell density-dependent apoptosis in HL-60 cells? It is reported that TNF-α induced apoptosis in these cells. However, we noted that a high concentration of TNF-α (100 ng/ml) induced only a weak smear-formed DNA breakdown with minimal ladder formation even after 2 days of incubation (data not shown); this would suggest that TNF receptor (TNFR)-mediated pathways may not be involved in the induction of cell density-dependent apoptosis in HL-60 cells. This speculation is further supported by our observation that this apoptosis was effectively blocked by the overexpression of Bcl-2 protein, because it is reported that Bcl-2-overexpressing HL-60 cells are sensitive to TNFR-mediated apoptosis (18). Similarly, the Fas-mediated pathways would also not be involved, since anti-Fas antibody did not cause any DNA breakdown at all (data not shown). It is known that TNFR- and Fas-mediated apoptosis are mediated by ICE-dependent pathway. Our observation that neither an ICE nor CPP-32 inhibitor blocked the cell density-dependent apoptosis in HL-60 cells confirms our speculation.

TGF-β1 is also known as an apoptosis-inducing cytokine, and it has been reported to induce apoptosis in hematopoietic cells.
fibroblasts (21), and epidermal cells (22–24). However, it is not yet understood how the signals for this induction are transmitted through the TGF-β receptor (TGF-βR). Recently, a novel mitogen-activated kinase kinase kinase and mitogen-activated kinase kinase were cloned that mediate the signals from TGF-βR (25, 26). Because mitogen-activated kinase kinase-mediated signaling pathways are reported to be involved in the induction of apoptosis in rat PC-12 cells (27) and also in mink Mv1Lu cells (28), it seems likely that TGF-β1 induces apoptosis by activating these pathways.

However, we observed a completely unexpected result: TGF-β1 strongly inhibited the induction of the cell density-dependent apoptosis in HL-60 cells. There is only one report mentioning the anti-apoptotic effect of TGF-β1, where it is shown that the apoptosis of peripheral T cells in T cell receptor-transgenic mice (29) was inhibited by the coexistence of TGF-β and interleukin-2. But even in this case, TGF-β added alone without interleukin-2 induced apoptosis (29). Our result is thus the first that clearly illustrates the sole contribution of TGF-β1 to apoptosis inhibition. Furthermore, this anti-apoptotic effect of TGF-β1 seems to be specific for the cell density-dependent apoptosis in HL-60 cells, because it did not inhibit but, instead, enhanced the actinomycin D-induced apoptosis in these cells (data not shown).

The mechanism of this inhibition by TGF-β1 in HL-60 cells is a matter of great interest. Decreased production of autocrine apoptosis-inducer by TGF-β1 was ruled out (Fig. 6F), and its antagonistic effect on the apoptosis inducer was confirmed (Fig. 6G). These results suggest two possibilities for the mechanism of the inhibitory effect on cell density-dependent apoptosis. One is the rapid interaction of intracellular...
signaling events triggered by TGF-β1 with those by the apoptosis inducer. The other is the more delayed effect of TGF-β1, probably via synthesis of certain anti-apoptotic proteins. According to our preliminary studies, TGF-β1 had no effect on the protein expression of well-known anti-apoptotic genes, including bcl-2, bcl-xL, and bcl-1. Further investigations from the standing point of signaling transduction and also new gene induction are required.

It has also been reported that TGF-β1 was able to inhibit cell proliferation (30); this seems to be achieved by the arrest of the cell cycle progression (31). In fact, we observed inhibition of proliferation (decrease in cell cycle progression speed) in human leukemic MO7e cells by TGF-β1 (data not shown). We demonstrated earlier, however, that TGF-β1 had no effect on proliferation speed (progression of cell cycle) in HL-60 cells despite its potent inhibitory effect on apoptosis. These findings together indicate that TGF-β1 affects the cell cycle progression or apoptosis induction according to the cell types and also suggest that the signaling pathways for cell cycle arrest and those for apoptosis inhibition are independently regulated.

We showed that the overexpression of Bcl-2 protein strongly inhibited the induction of cell density-dependent apoptosis in HL-60 cells. We also showed that the conditioned medium of Bcl-2 overexpressing HL-60 cells had as well an activity to induce apoptosis in parental cells, indicating that Bcl-2 protein did not inhibit the release of the apoptosis-inducing factor but, rather, antagonized the intracellular signaling pathway for apoptosis induction. It is also of interest whether other Bcl-2 family proteins, including Bcl-x and Bax, can inhibit HL-60 cells spontaneous apoptosis.

Whether cell density-dependent apoptosis is a phenomenon specific to HL-60 cells or is more generalized is an interesting point. We also investigated the existence of cell density-dependent apoptosis using two other human leukemic lines, U937 cells and MO7e cells, and we observed that MO7e cells underwent apoptosis in the course of proliferation when the cell density rose above 3 × 10⁶/ml. Further investigations from the standing point of signaling transduction and also new gene induction are required.

Cell density-dependent apoptosis is physiologically of great importance not only in vitro but also in vivo, since it may contribute to the homeostasis of blood cell production via a negative feedback regulator of cell proliferation. Mechanistic study of cell density-dependent apoptosis may also lead to the development of a novel therapy for hematological malignan-
cies. Further studies are required to prevent the uncontrolled proliferation of tumor cells by the application of cell density-dependent apoptosis.

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