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Overexpressed Mitochondrial Hinge Protein, a Cytochrome *c*-Binding Protein, Accelerates Apoptosis by Enhancing the Release of Cytochrome *c* from Mitochondria

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Holocytochrome *c* released from mitochondria has been revealed to be one of the contributors of apoptosis. To investigate how the cytochrome *c* protein is released from mitochondria, we examined the effects of overexpression of the hinge protein, a cytochrome *c*-binding protein, or cytochrome *c* on apoptosis by introducing their cDNAs under a constitutive promoter. Overexpression of the cytochrome *c* and hinge protein mRNAs was confirmed by Northern blotting, although marked accumulation of the cytochrome *c* protein was not observed. In transfectants of the hinge protein gene as well as cytochrome *c* gene, apoptosis was accelerated as judged by FITC-conjugated Annexin V binding to the cell surface and DNA fragmentation. In addition, enhancement of the release of cytochrome *c* into cytosol was demonstrated in these transfectants by a subcellular fractionation experiment, followed by Western blotting. These findings suggest that the release of the cytochrome *c* protein from mitochondria is regulated by the hinge protein involved in the respiratory chain in the apoptotic process.

Apoptosis or programmed cell death plays essential roles in tissue homeostasis and developmental and pathological elimination of redundant or excess cells in multicellular organisms (1). The apoptotic process involves a characteristic set of morphological events, which include plasma membrane blebbing, cell shrinkage, chromatin condensation, nuclear fragmentation, and cell fragmentation (2, 3). DNA fragmentation is a biochemical indicator of apoptosis because oligonucleosomal DNA degradation appears as a "ladder" of DNA bands in agarose gel electrophoresis (4). In addition, Annexin V bound to phosphatidylserine of the cell surface is another indicator, because an early event of apoptosis results in exposure of phosphatidylserine to the cell surface (5, 6).

Reports indicating the contributions of mitochondria to apoptosis have been accumulating (7-9). The mitochondrial fraction is required to induce nuclear apoptosis *in vitro* (7). Anti-apoptotic Bcl-2 transfers Raf-1 to mitochondria to inhibit apoptosis (10). In addition, decrease of the mitochondrial transmembrane potential has been observed before a morphological change of nucleus, and release of a kind of protease from mitochondria was proposed (9, 11). Moreover, holocytochrome *c* has been identified as a factor involved in nuclear apoptosis *in vitro* (12). Further study revealed that cytochrome *c* is essential for the proteolytic activity of Apaf-1 which in turn activates Caspase-3 (13). The release of cytochrome *c* is inhibited by Bcl-2 or Bcl-x_L (14-16). Since Bcl-x_L binds to cytochrome *c*, Bcl-x_L or Bcl-2 may inhibit release of cytochrome *c* by capture of cytochrome *c* itself, resulting in inhibition of apoptosis (16). These findings high-light the novel functions of mitochondria in apoptosis.

Cytochrome *c* is a respiratory protein located on the surface of the mitochondrial inner membrane facing the intermembrane space, and plays a role in the transfer of electrons from the cytochrome bc_1 complex to cytochrome *c* oxidase (17, 18). This protein is encoded by a nuclear gene and translated on cytosolic ribosomes as apocytochrome *c*. Apocytochrome *c* is subsequently translocated into mitochondria where a heme group is attached covalently to form holocytochrome *c*. Cytochrome *c* is bound to an acidic component, termed the hinge protein (19), of the cytochrome bc_1 complex on the inner membrane. Thus, cytochrome *c* is located on the surface of the inner membrane via the hinge protein, suggesting that the hinge protein contributes to

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the location of cytochrome *c*. The cDNA clone of the human hinge protein was cloned by us (20).

In this study, we introduced a cDNA of the hinge protein in a constitutive expression vector into a murine promyeloid cell line and found that overexpression of the hinge protein accelerated apoptosis induced by withdrawal of IL-3 and treatment of Staurosporine, a potential inhibitor of protein kinases. This finding suggests that the release of cytochrome *c* is regulated by the hinge protein of the respiratory chain.

MATERIALS AND METHODS

Plasmid constructs and transfection. Plasmid vector pEF-BOS (21) was used as the expression vector for murine cells. The human cytochrome *c* cDNA (22) and the human hinge protein (20) cDNA of full-length were amplified by PCR and then cloned into the *Xba*I and *Nco*I-*Xba*I sites of modified pEF-BOS, respectively, as described (23). The nucleotide sequence of the insert was confirmed by nucleotide sequencing. The plasmid containing rat *bcl-2* cDNA was described in our earlier report (23). These constructs were co-introduced with pSTneoB into FDC-P1 cells by electroporation as described (23). After electroporation, the cells were cultured in microplates for selection by Geneticin (GIBCO BRL), and at the same time, cloned by the limiting dilution method.

Northern blot analysis. Total RNA was isolated from cells by using ISOGEN (Nippon Gene, Toyama) and subjected to formaldehyde/agarose gel electrophoresis. After blotting to a nylon membrane, Hybond-N^+ (Amersham), hybridization was performed in a Rapid-hyb buffer (Amersham) in a high stringent condition Probes were synthesized with DNA polymerase I (Klenow fragment) using purified DNA fragments corresponding to the full-length of coding regions of human cytochrome *c* and the human hinge protein as templates in the presence of [α-^{32}P]dCTP. Signals were detected with a bioimaging analyzer, Fujix BAS 1500 (Fuji Photo Film, Tokyo) and visualized with Fujix Pictrography 3000 (Fuji Photo Film, Tokyo).

Cell culture and induction of apoptosis. The murine promyeloid cell line, FDC-P1 was maintained in RPMI 1640 medium (Nissui, Tokyo) supplemented with 10% fetal bovine serum (FBS) and 5% WEHI-3B-conditioned medium as a source of IL-3 at 37°C in a humidified atmosphere of 5% CO_2 (24). Apoptosis of FDC-P1 or its transfectants were induced by depletion of IL-3 as described (25) or by adding a protein kinase inhibitor, Staurosporine (50 nM). Cells surviving were counted by the Trypan blue exclusion method at appropriate periods.

Flow cytometric analysis of Annexin V binding. The cells were washed twice with phosphate buffered saline (PBS) and suspended in the saline at 1×10^6 cells/ml. According to the instructions of the Apoptosis detection kit (R&D systems, Minneapolis), the cells were treated with a mixture of propidium iodide (PI) and FITC-Annexin V. More than 40,000 cells were analyzed with a flow cytometer EPICS ELITE ESP (Coulter, Florida). After analysis by quadrant statistics

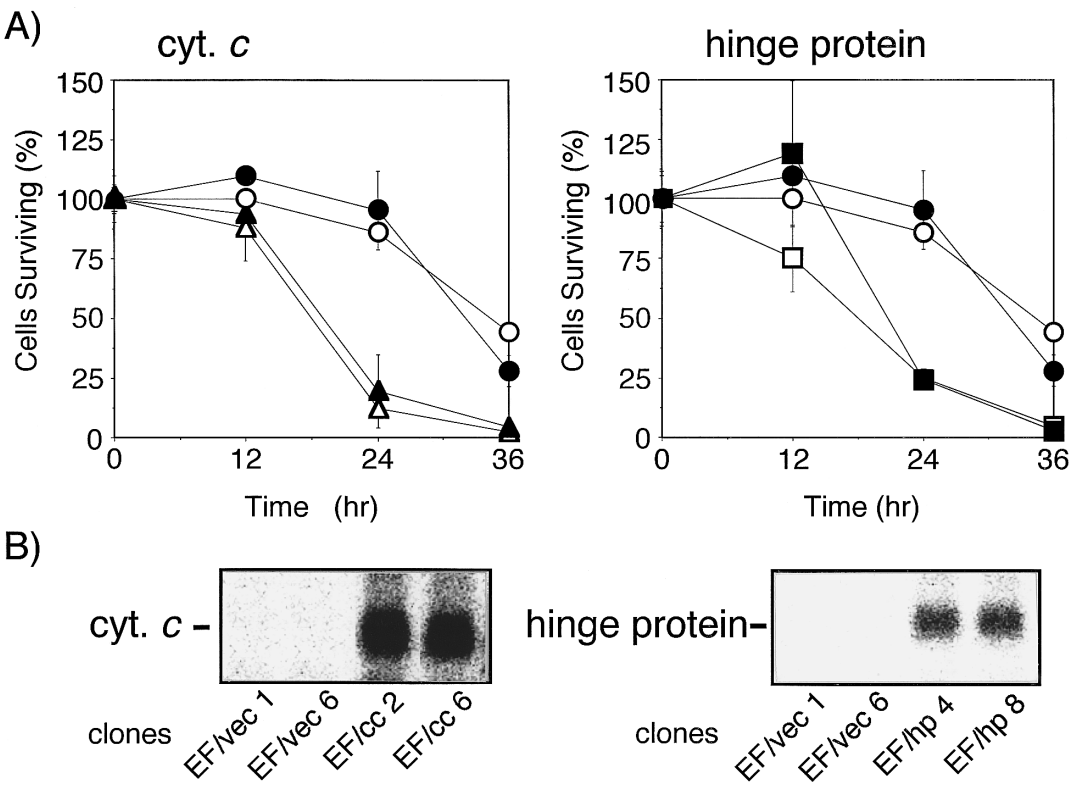

FIG. 1. Cells surviving after a depletion of IL-3 in transfectants with cytochrome *c* and the hinge protein. (A) Transfectant clones of FDC-P1 with plasmids containing cytochrome *c* cDNA (left, EF/cc2 △ and EF/cc6 ▲) and hinge protein cDNA (right, EF/hp4 □ and EF/hp8 ■) were counted by the Trypan blue exclusion method at the times indicated after depletion of IL-3. Control transfects (EF/vec1 ○ and EF/vec6 ●) were prepared by transfecting the parental cells with the vector without any insert. (B) Expressed levels of mRNAs of cytochrome *c* and hinge protein were examined by Northern blot analysis by using total RNA (5 μg). Probes were ^{32}P-labeled fragments of each full-length coding region of the cDNAs: (left) cytochrome *c* and (right) hinge protein indicate the position of each mRNA, respectively.

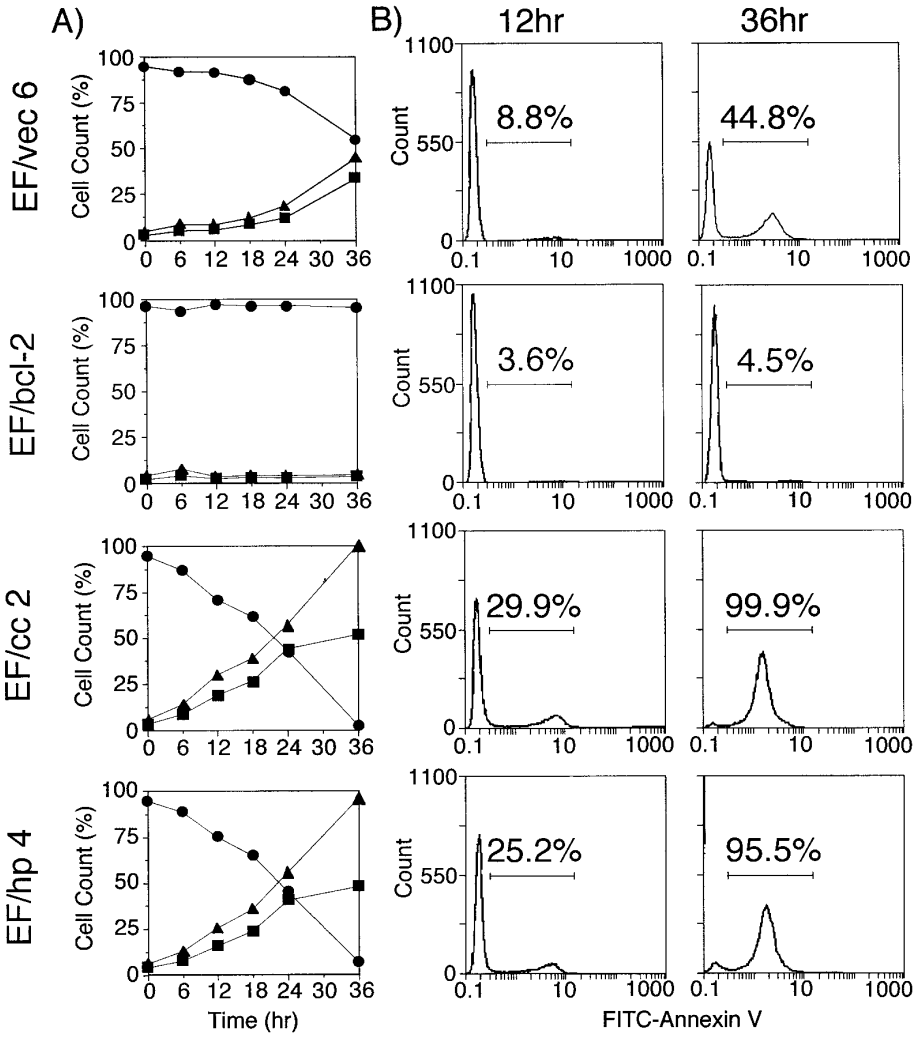

FIG. 2. Flow cytometric analysis of transfectants with cytochrome *c* and the hinge protein. Exposure of phosphatidylserine on the surface of early apoptotic cells was determined by bound FITC-Annexin V. After culture for the indicated periods without IL-3, the transfectants were washed and treated with FITC-Annexin V and propidium iodide (PI) to be stained, and then more than 40,000 cells were analyzed with a flow cytometer, EPICS ELITE ESP. (A) The analysis by quadrant statistics for determination of apoptosis showed the ratio of intact cells (both negative for FITC and PI)(●), FITC-Annexin V-positive cells (▲), and PI-positive cells (■). EF/vec6, EF/bcl-2, EF/cc2, and EF/hp4 indicate transfectant clones with empty vector, rat *bcl-2*, human cytochrome *c*, and the human hinge protein cDNAs, respectively. (B) Examples of the profiles showing FITC-Annexin V-positive cells. The cut-off values given as a percentage in the insets were used in A. 12 and 36 hr indicate the periods after IL-3 removal in each clone corresponding to A.

for determination of FITC-Annexin V-positive and PI-negative cells were considered as early apoptotic cells and PI-positive cells as dead cells.

Determination of DNA fragmentation. Fragmented DNA was prepared and detected as described (26). Briefly, cells were harvested and treated with 1% Triton X-100. After removal of intact nuclei by centrifugation, fragmented DNAs were treated with phenol and recovered by 2-propanol precipitation, followed by 1.2% agarose gel electrophoresis. The DNA was stained with ethidium bromide and visualized under ultraviolet radiation.

Determination of cytochrome c release in cytosol. The release of cytochrome *c* from mitochondria into cytosol in the apoptotic cells was examined by subfractionation of the cell. Transfectants, 1×10^8 cells, were harvested by centrifugation at $300 \times g$ for 5 min at 4°C. The cell pellets were washed once with ice-cold PBS and resuspended with 1 ml of buffer A (20 mM Hepes-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonylfluoride (PMSF) containing 250 mM sucrose). The cells were broken by 20 strokes with a Dounce glass homogenizer, and then the homogenates were centrifuged at $750 \times g$ for 10 min at 4°C to remove nuclei and unbroken cells. The resultant supernatant was centrifuged at $15{,}000 \times g$ for 15 min at 4°C to remove mitochondria, and the final supernatant was used as the cytosol fraction for Western blot analysis. Artificial disruption of mitochondria by this treatment was monitored by Western blotting using polyclonal antibody against the E2 subunit of the pyruvate dehydrogenase complex, which is located in the mitochondrial matrix.

The cell lysate was prepared to determine the amount of cytochrome *c* in the transfectants. Transfectants, 1×10^7 cells, were washed once with PBS and lysed with 0.5 ml of lysis buffer (1.0% NP-40, 142.5 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes (pH 7.2), 1 mM EGTA, 0.2 mM PMSF, and 0.7 μg/ml Pepstatin) for 10 min, and the

soluble fraction was obtained by centrifugation at 15,000 × *g* for 20 min at 4°C. The soluble fraction was used as cell lysate in Western blotting. The resulting cytosolic protein or cell lysate (10 μg of protein in each case) was separated by SDS-PAGE (15% gel) followed by transfer to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with PBS containing 10% FBS for 1 hr at 37°C, and was probed with anti-cytochrome *c* monoclonal antibody (Pharmingen, San Diego, LA) (1:500) for 90 min at 37°C. After rinsing with a Western blot buffer (20 mM Tris-HCl, pH 7.4, 136 mM NaCl, 0.2% Tween 80), the membrane was treated with 1/10,000 diluted horseradish peroxidase-labeled anti-mouse IgG polyclonal Fab fragment for 90 min at 37°C. After washing the membrane, bands of cytochrome *c* were detected by chemiluminescence (DuPont NEN, Boston, MA).

RESULTS AND DISCUSSION

Overexpression of cytochrome c and the hinge protein accelerates apoptosis. Release of cytochrome *c* from mitochondria is a step in the activation of Caspase-3 in undergoing apoptosis (13). Since it is unknown whether overexpression of cytochrome *c* accelerates apoptosis, we introduced cDNA of apocytochrome *c* under a constitutive promoter into murine promyeloid cell line FDC-P1, in which apoptosis was easily induced by withdrawal of IL-3 and addition of Staurosporine. At the same time, cDNA of the hinge protein was transfected into the same cells to investigate the regulatory mechanism of the release of cytochrome *c*. As shown in Fig. 1, clones expressing mRNA of apocytochrome *c* or the hinge protein were obtained and the clones were designated EF/cc1-10, EF/hp1-10 and EF/Vec1-10 for the transfectants of cytochrome *c*, the hinge protein and the empty control vector, respectively. Since the human genes were introduced, endogenous murine mRNAs for cytochrome *c* and the hinge protein were not detected as seen in Fig. 1. When IL-3 was removed from the medium, cell death of the transfectants with the hinge protein as well as cytochrome *c* was accelerated as judged by the Trypan blue exclusion method (Fig. 1).

To confirm that the cells died via an apoptotic process, a flow cytometric analysis was applied by using FITC-conjugated Annexin V. In an early event of apoptosis, Annexin V is known to bind specifically to phosphatidylserine exposed to the cell surface (6, 7). Fig. 2 shows the time course of Annexin V binding. In the transfectants with the hinge protein and cytochrome *c*, the binding was clearly observed at an early time after depletion of IL-3, indicating that the accelerated cell death was apoptosis. The other transfectant clones showed nearly the same results.

As another indicator of apoptosis, the extent of DNA fragmentation was examined as described in MATERIALS AND METHODS. Fig. 3 shows electrophoretic patterns of the extracted DNA, indicating the increase of fragmented DNA in the transfectants with cytochrome *c* and the hinge protein over the time course. Moreover, the fragmentation of DNA was accelerated in the transfectants with the hinge protein and cytochrome *c* cDNAs in apoptosis induced not only by IL-3 depletion but also by Staurosporine (Fig. 3, Panel B). The other clones also showed nearly the same results.

Therefore, we concluded that the overexpression of cytochrome *c* and the hinge protein accelerated apoptosis.

Overexpression of cytochrome c and the hinge protein enhances the release of cytochrome c from mitochondria. The content of cytochrome *c* was examined by Western

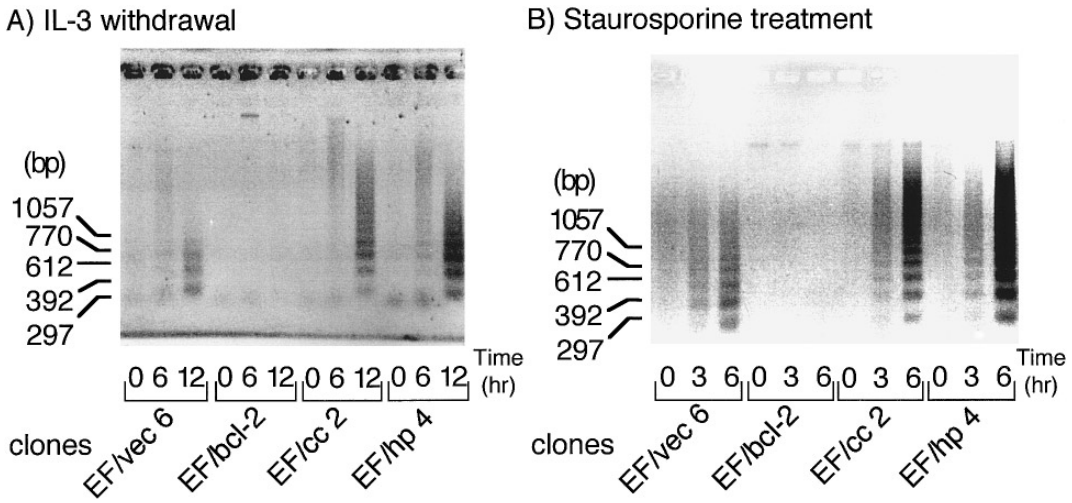

FIG. 3. DNA fragmentation after depletion of IL-3 or treatment with Staurosporine. Transfectants were harvested after depletion of IL-3 (A) at 0, 6, or 12 hr and addition of Staurosporine (50 nM) (B) at 0, 3, or 6 hr as indicated. The fragmented DNA was prepared, electrophoresed, and visualized as described in MATERIALS AND METHODS. EF/vec6, EF/bcl-2, EF/cc2, and EF/hp4 indicate clones with empty vector, rat *bcl-2*, human cytochrome *c*, and the human hinge protein cDNAs, respectively. The size of standard DNA fragments is given on the left.

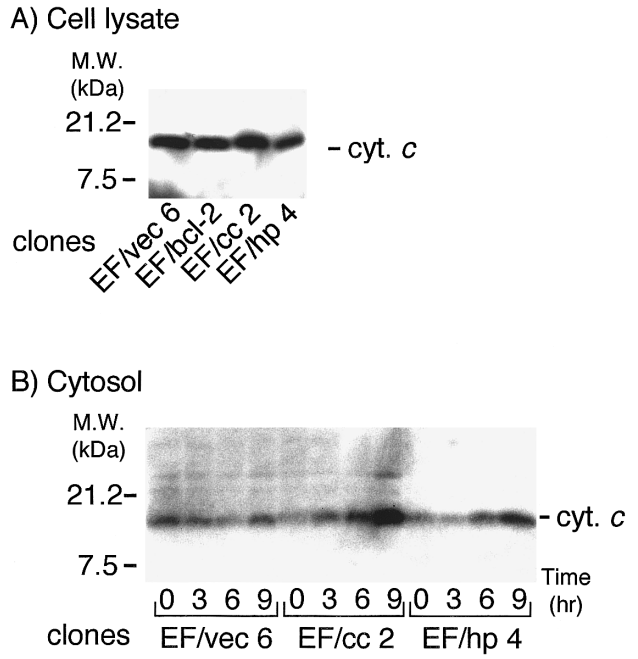

FIG. 4. Western blot analysis of cytochrome *c*. (A) Cell lysate (10 μg total protein) from each transfectant was analyzed by Western blotting. EF/vec6, EF/bcl-2, EF/cc2, and EF/hp4 indicate transfectant clones with empty vector, rat *bcl-2*, human cytochrome *c*, and the human hinge protein, respectively. The position of cytochrome *c* is indicated as cytochrome *c*. (B) Cytosolic fractions from each transfectant were obtained at 0, 3, 6, and 9 hr as indicated after IL-3 withdrawal and analyzed by Western blotting analysis as described in MATERIALS AND METHODS. Note that the exposure time of chemiluminescence was longer than that shown in A.

blotting using anti-cytochrome *c* monoclonal antibody. Unexpectedly, the cytochrome *c* protein was slightly accumulated in the transfectants with cytochrome *c* cDNA (Fig. 4A) although overexpressed mRNA for cytochrome *c* was detected (Fig. 1). This is probably because the capacity of cytochrome *c* inside mitochondria is limited and excess cytochrome *c* may be degradated in the cytosol or mitochondria. The hinge protein and Bcl-2 did not affect the content of cytochrome *c* either.

The release of cytochrome *c* from mitochondria was examined by a subcellular fractionation experiment as described in MATERIALS AND METHODS. Artificial disruption of mitochondria during the cell fractionation procedure was monitored by an amount of a mitochondrial matrix protein, the pyruvate dehydrogenase complex E2 subunit by Western blotting using anti-the E2 subunit, and the amounts of the E2 subunit detected in the supernatant fractions were in nearly propotion to the background levels of cytochrome *c* shown in Fig. 4B, at 0 and 3 hr (data not shown). When IL-3 was removed, the amounts of cytochrome *c* of the supernatant fractions markedly increased in the transfectants with cytochrome *c* and the hinge protein (Fig. 4, panel B at 6 and 9 hr). Approximately 10% of total cytochrome *c* was released into cytosol as judged by densitometric analysis (data not shown). Thus, the release of the cytochrome *c* protein from mitochondria into cytosol was enhanced by the expression of the mRNAs of cytochrome *c* and the hinge protein (Fig. 4B, EF/cc2 and EF/hp4, at 6 and 9 hr).

Probably because excess cytochrome *c* in the intermembrane space could be easily released from mitochondria into cytosol, the overexpression of the cytochrome *c* gene could accelerate apoptosis even with slight accumulation of the cytochrome *c* protein. More interestingly, the overexpression of the hinge protein accelerated apoptosis by enhancement of the release of cytochrome *c*. Since the hinge protein is a cytochrome *c*-binding protein, it is considered to function as a regulator of respiration. This study proposes that the hinge protein inside mitochondria contributes to apoptosis by regulating the release of cytochrome *c* from mitochondria. Other components of the respiratory chain may contribute to the regulation of apoptosis by capture or release of cytochrome *c*. Further study is necessary to elucidate the molecular mechanism regulating the release of cytochrome *c* as a signal of apoptosis.

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