Inhibition of apoptosis by the expression of antisense *Nedd2*

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Received 27 April 1995; revised version received 23 May 1995

Abstract Nedd2 belongs to a family of mammalian cysteine proteases which share similarity with the *Caenorhabditis elegans* cell death protein, Ced-3. Overexpression of *Nedd2* has been shown to induce apoptosis in mammalian cells but in the absence of a specific known inhibitor, it remains to be seen whether this represents cytotoxic effects of the Nedd2 protease or the specific activation of an apoptotic pathway. The present work shows that the factor-dependent cell line FDC-P1 expressing mouse antisense *Nedd2* mRNA, exhibits significant inhibition of cell death upon removal of the cytokines, thus providing evidence for a direct role of *Nedd2* in mediating apoptosis.

Key words: ICE-like protease; Nedd2; Programmed cell death; FDC-P1 cells

1. Introduction

Apoptosis or programmed cell death (PCD) is an active process of cell suicide characterised by a distinct set of morphological events involving plasma membrane blebbing, loss of cell volume, nuclear condensation, and fragmentation of DNA at nucleosomal intervals [1,2]. Cell death by apoptosis is essential to remove unwanted cells and to regulate cell number during development and homeostasis in metazoans [3,4]. Much of the recent information on the molecular regulation of apoptosis has come from the genetic analysis of the nematode *Caenorhabditis elegans* [5]. Two genes, *ced-3* and *ced-4*, required for the developmental cell death in the worm have been cloned in the recent years [5]. While *ced-4* encodes a putative calcium binding protein, nothing is known about its function and no mammalian homologues of the Ced-4 protein have yet been discovered [6]. On the other hand, cloning of *ced-3* indicated that its product is similar to interleukin-1β converting enzyme (ICE) [7], a cysteine protease required for the proteolytic activation of IL-1β [8–10], and the product of a developmentally regulated mouse gene *Nedd2* [11,12]. Another Ced-3 homologue named CPP32 has been recently cloned [13]. In addition, prICE, a cysteine protease activity, distinct from ICE has been identified in apoptotic cell extracts [14]. There is now growing evidence suggesting a central role for ICE-family of cysteine proteases as effectors of PCD [15].

The evidence for the role of these ICE-like mammalian proteins in apoptosis is largely derived from the observation that the overexpression of *ICE*, *Nedd2* and *CPP32* induces apoptosis in eukaryotic cells [12,13,16,17]. These experiments are inconclusive since the overexpression of a protease might have cytotoxic effects irrespective of its role in the mediation of apoptosis. More direct evidence for a role of ICE and prICE in apoptosis has come the from the inhibitor studies. For example, CrmA, a viral serpin inhibitor of ICE [18] has been shown to inhibit neuronal cell death induced by deprivation of nerve growth factor [19]. However, specificity of CrmA inhibition is unknown and it is possible that it also acts on other ICE-like proteases. Try-Val-Ala-Asp-chloro-methylketone (YVAD-CMK), an ICE and prICE inhibitor is also known to inhibit apoptosis associated morphological changes in an in vitro system [14,20]. Yet again, YVAD-CMK is not specific for any one of these proteases and probably acts on all ICE homologs requiring Asp residue in P1 position of the substrate. From the studies published so far, it is unclear whether one or more of the ICE-like proteases are required in a single apoptotic pathway or whether these proteins are functionally redundant.

Despite much in vitro experimental evidence implicating ICE in apoptosis, mice lacking functional *ICE* gene are overtly normal and show no apparent defect in dexamethasone- or ionising radiation-induced apoptosis of thymocytes [21]. However, *ICE*(−/−) mice are resistant to apoptosis induced by Fas antibody [22]. This suggests that the in vivo role of ICE may be limited to Fas mediated apoptosis and thus, various ICE-like proteases may act in different apoptotic pathways. To establish, which of the ICE-like proteins are required in a particular apoptotic pathway, experiments involving functional inactivation of the individual members would be required. This can be achieved by gene targetting, as has been done with ICE, and by using specific inhibitors of individual proteins.

In the absence of a known specific inhibitor of Nedd2 function, in the present work, we have used antisense mRNA expression as a means to modulate the levels of Nedd2 to study whether it plays a direct role in apoptosis. We show that expression of *Nedd2* antisense mRNA in a factor-dependent murine hematopoietic cell line FDC-P1 [23], significantly inhibited the apoptosis of these cells under cytokine deprived conditions. This provides a compelling evidence that Nedd2 protein is required for apoptosis in FDC-P1 cells, and its inhibition alone can suppress cell death.

2. Materials and methods

2.1. Expression constructs and transfection of cells

The construction of *Nedd2* expression vectors has been described before [12]. In pCXN2-N2 (*Nedd2* sense expression construct), the cDNA containing the entire coding region of *Nedd2* was released from clone pMS N2.4 and inserted downstream of the chicken β-actin promoter in pCXN2 vector [24]. The antisense construct (pCXN2-N2AS) contained a 1.0 kb *Sal*I fragment of the clone pMS N2.4, representing

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Abbreviations: PCD, programmed cell death; ICE, interleukin-1β-converting enzyme; GM-CSF, granulocyte/macrophage colony stimulating factor; IL-3, interleukin-3; DMEM, Dulbecco's modified Eagle's medium; IMDM, Iscove's modified Dulbecco's medium; FCS, fetal calf serum; kb, kilobase(s).

SSDI 0014-5793(95)00602-8

the 5'-end of the mouse *Nedd2* mRNA, in antisense orientation [12]. FDC-P1 cells were routinely maintained in DMEM supplemented with 10% fetal calf serum (FCS) and 40 units/ml of murine GM-CSF. For each transfection, 4 μg of plasmid DNA in 0.5 ml OPTIMEM (Life Technologies) was mixed with 10 μl of Lipofectamine (Life Technologies) in 0.5 ml Optimem and incubated at room temperature for 30 min. FDC-P1 cells (10^6/transfection) were washed once in Optimem, resuspended in DNA/Lipofectamine mix, supplemented with 40 units/ml GM-CSF, and plated into a 6-well dish. After 6 h incubation at 37°C, each well was fed with 3 ml of Optimem containing 20% FCS and 40 units/ml GM-CSF. The following day, cells were pelleted, washed and plated in DMEM supplemented with 10% FCS, 40 units/ml GM-CSF and 1 mg/ml G-418. Transfected cells were selected for antibiotic resistance for 2-weeks prior to cloning. Cloning of the transfected cells was done in methyl cellulose/IMDM semisolid medium [25]. Approximately 200 cells were mixed with methyl cellulose/IMDM mix supplemented with 10% FCS, 1 mg/ml G-418 and 50 units/ml GM-CSF, and plated in 60 mm dishes. Well isolated colonies were picked after 7–14 days incubation and maintained in DMEM with FCS, GM-CSF and 1 mg/ml G-418.

2.2. RNA isolation and analysis

Poly(A)$^+$ RNA was isolated from cultured cells using the Fast Track kit (Invitrogen) or by oligo(dT) batch absorption [26]. Approximately 2.0 μg of poly(A)$^+$ RNA samples were electrophoresed on 1.2% agarose/2.2 M formaldehyde gels, transferred to Biodyne A membrane (Pall) and hybridized to probes labelled with [α-^{32}P]dCTP by random priming using Klenow fragment of DNA polymerase [26]. Mouse *Nedd2* probe was a 2. 2 kb *Bam*HI/*Xho*I fragment of the clone MS N2.4 representing the entire coding region of the cDNA [10]. Human *NEDD2* (*Nedd2* and *NEDD2* are approved gene symbols for mouse and human homologues, respectively) probe was derived as a 0.8 kb *Eco*RI fragment of the clone Hb N2.3 [27].

2.3. Cell death experiments

Cells were washed in DMEM supplemented with FCS three times and resuspended at 2×10^6 cells/ml in the same medium. One-hundred μl aliquots were incubated in 96-well plates for the desired length of time. In the control experiments, cells washed in a similar way were resuspended in medium containing 40 units/ml of GM-CSF or 100 units/ml IL-3 to account for the cell death which may occur during washing procedures. Equal volumes of cell suspension were mixed with 0.8% Trypan blue and dye positive and negative cells were counted using a hemocytometer. In these experiments, at time zero (soon after the washing steps), typically more than 90% cells were viable as determined by Trypan blue dye exclusion. In all cell death experiments, cells were examined for apoptosis-associated morphological changes and DNA fragmentation, as described previously [12]. To adapt FDC-P1 transfectants to IL-3, cells were grown for a week in the medium supplemented with 40 units/ml GM-CSF and 100 units/ml IL-3, then switched to medium with IL-3 alone. Cells were maintained in IL-3 medium for atleast 2 weeks prior to cell death experiments.

3. Results and discussion

Factor-dependent hematopoietic cell lines have been extensively used to study PCD (e.g. [28]). Upon removal of cytokines, these cells undergo rapid cell death by apoptosis. Since there is no information available on the expression of *Nedd2* in hematopoietic cells, we first examined a number of human cell lines by Northern blot analysis for *NEDD2* mRNA. All cell types analyzed expressed moderate levels of the 4.2 kb human *NEDD2* transcript (Fig. 1). These cell lines represented several human myeloid and lymphoid lineages and included B-cell lymphoblasts (Balm), erythroleukemia (HELdr, HEL 900), mast cell (HMC-1), T-cell lymphoblasts (Molt4, CCRF-CEM), megakaryocytes (Mo7e), myelomonocytes (RC2A) and promyelocytes (HL60). This widespread expression in hematopoietic cells suggests that *Nedd2* has an important role as a mediator of apoptosis in blood cells.

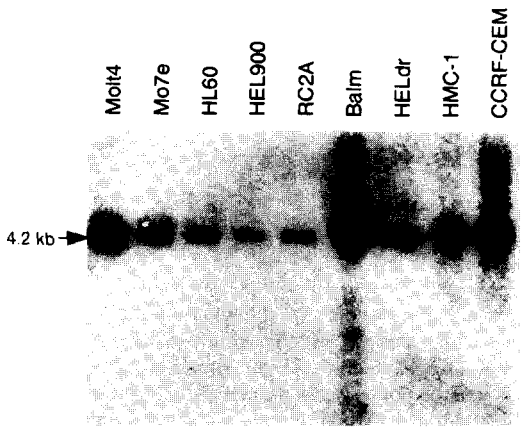

Fig. 1. Expression of *NEDD2* in various human hematopoietic cell lines. Each lane contained approximately 2 μg of poly(A)$^+$ RNA isolated from the indicated cell line. Blots were hybridized with a human *NEDD2* cDNA probe [27] which detected a single 4.2 kb transcript in all cell lines. The quality and quantity of RNA preparions were determined by ethidium bromide staining and hybridization to a glyceraldehyde 3-phosphate dehydrogenase probe (not shown). Data are derived from two different gels.

Murine myeloid progenitor cell line FDC-P1, which also expressed moderate levels of *Nedd2* mRNA (Fig. 2) was chosen for the antisense studies using mouse *Nedd2* cDNA expression constructs, because of its apoptosis response has been well characterized [29,30]. This cell line can be maintained with either GM-CSF or IL-3 and upon removal of the factor undergoes apoptosis within 1–2 days [29,30]. FDC-P1 cells were transfected by lipofection with a mammalian expression vector (pCXN2), the vector carrying *Nedd2* cDNA containing entire coding region in sense orientation (PCXN2-N2), or 5'-1.0 kb fragment of the *Nedd2* cDNA in antisense orientation (pCXN2-N2AS). Transfected cells were selected with G-418 for two weeks and antibiotic resistant cells were then cloned by plating into semisolid medium. In methyl cellulose semisolid medium, the colony size and morphology of antisense and vector-transfected cell were similar; however, cells transfected with *Nedd2* sense construct formed much smaller colonies (10–20% in diameter). But in liquid cultures the growth rates of cells transfected with either vector, sense or antisense were comparable. The reasons for these variations in growth characteristics of *Nedd2* sense-transfected cells are not known.

Several individual clones were analysed by Northern blotting for the expression of introduced cDNA. Comparable levels of expression was detected in various clones transfected with a particular expression construct, although the expression of mRNA from introduced plasmids was always far greater for the antisense than the sense (Fig. 2). This is consistent with the fact that overexpression of *Nedd2* induces apoptosis in various cell lines [12,16] and FDC-P1 cells expressing *Nedd2* over a certain threshold are likely to undergo apoptosis. Thus, antibiotic selection for the transfected cells would favour transfectants which express relatively low levels of *Nedd2*. In all the sense-transfected clones, the ratio of transfected versus endogenous expression was 2–3, which probably reflects a measure of tolerance of these cells. The *Nedd2* antisense-transfected cells on the other hand, show levels of RNA expression atleast 20 times the endogenous *Nedd2* transcript (Fig. 2).

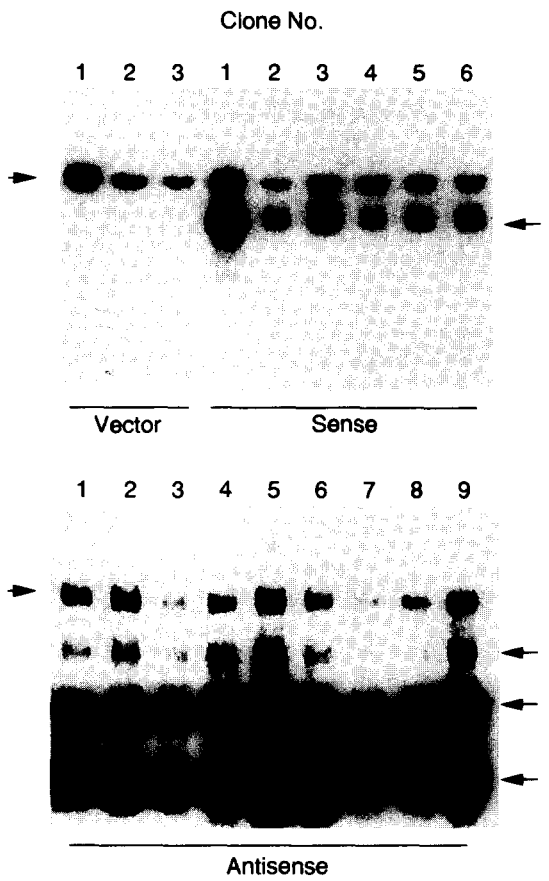

Fig. 2. Expression of *Nedd2* in transfected FDC-P1 cells. Cloned transfected cells were used for the isolation of poly(A)$^+$ RNA. Each lane contains approximately 2 μg of poly(A)$^+$ RNA. Blots were hybridized with a mouse *Nedd2* probe. The arrow on the left indicate the 3.5 kb endogenous transcript while the ones on the right indicate the transcripts derived from introduced expression constructs. The transfected *Nedd2* sense construct produces a transcript of approximately 2.3 kb, while the antisense construct generates a 1.3 kb mRNA species. Additional transcripts seen in antisense tranfected cells most likely result from aberrant splicing and/or termination [12].

Several vector-, sense- and antisense-transfected clones were analysed for the cell death induced in the absence of factor and the results for some are shown in Fig. 3. In general, there was no significant variations between various clones derived from cells tranfected with an individual expression construct. Control FDC-P1 cells transfected with the empty vector showed typical response with most (>90%) cells undergoing apoptosis within 24 h of the removal of GM-CSF (Fig. 3A). The cells transfected with the sense *Nedd2* showed similar patterns of cell death when plated in factor-free medium. On the other hand, clones expressing antisense *Nedd2* showed significant inhibition of cell death under similar conditions (Fig. 3A). After 24 h in factor-free medium, while less than 10% of the vector-transfected control cells were Trypan blue negative, approximately 40% of the antisense expressing cells excluded the dye. Morphological observations confirmed that dye-excluding cells exhibited no characteristic features of apoptosis (data not shown). In all experiments, the cells washed and replated in GM-CSF containing medium did not show any significant cell death (data not shown). To analyse whether IL-3 dependent FDC-P1 cells show similar effects, transfected clones were adapted to IL-3 for at least two weeks and subjected to IL-3-free conditions. Again, the clones expressing antisense *Nedd2* showed significant inhibition of cells death (Fig. 3B). These results clearly indicate that *Nedd2* antisense expression suppresses/delays the apoptosis induced by the deprivation of either GM-CSF or IL-3 in FDC-P1 cells.

The levels of endogenous *Nedd2* mRNA did not appear to be significantly altered in the cells expressing antisense. It is therefore likely that antisense expression inhibits the translation of endogenous *Nedd2* mRNA. Because of the unavailability of a suitable antibody against Nedd2, it was not possible to check the levels of endogenous Nedd2 in antisense-transfected cells. Nevertheless, the data presented here provide a strong evidence for a direct role for *Nedd2* in apoptosis. It also suggests that modulation of *Nedd2* alone in FDC-P1 cells can

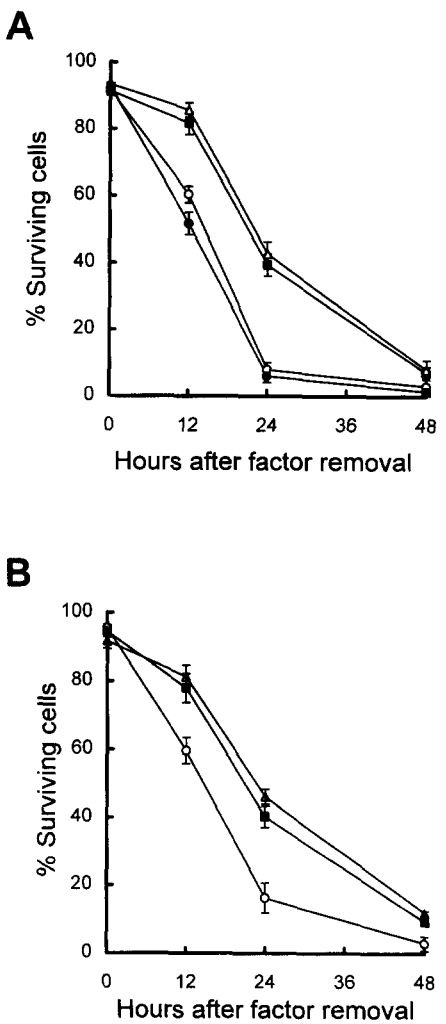

Fig. 3. Inhibition of cell death by antisense *Nedd2* expression. Tranfected cloned cell were maintained in either GM-CSF (A) or IL-3 (B) and subjected to cytokine-free conditions to induce apoptosis as described in section 2. At indicated time after plating in factor-free medium, cells were mixed with Trypan blue and both stained and dye excluding cells counted. Each experiment was done in triplicate and the data from a single experiment is plotted. Similar results were obtained in at least six independent experiments employing several different clones. Vector-transfected clone 2 (○); sense *Nedd2*-transfected clone 3 (●); antisense *Nedd2*- transfected clone 5 (■) and clone 9 (△).

inhibit cell death. In addition to *Nedd2*, low level *ICE* expression was also detected in FDC-P1 cells (data not shown). mRNA for *CPP32* has been shown to be highly expressed in several cell lines of hematopoietic lineages [14]. Since *Nedd2* cDNA sequence shares only partial similarity with *ICE* and *CPP32* [10,12,13], antisense *Nedd2* expression is unlikely to alter the levels of endogenous ICE and CPP32 proteins. In DNA and RNA blot analyses, probes derived from *Nedd2* cDNA, do not detect other homologues [12]. Furthermore, no changes in the ICE mRNA and protein levels were observed in FDC-P1 clones expressing the antisense *Nedd2* transcript (data not shown). Inhibition of apoptosis by the suppression of Nedd2 suggests that this protease constitutes a component of the cell death machinery in FDC-P1 cells. However, we cannot rule out the involvement of the other ICE family members as the expression of *Nedd2* antisense only partially inhibited the apoptosis of FDC-P1 cells.

An alternately spliced form of *Nedd2* mRNA which encodes a truncated form of protein, Nedd2s (Ich-1s in [16]) has been shown to act as a negative regulator of apoptosis in Rat-1 cells [16]. As the sequences of *Nedd2* and *Nedd2s* mRNA are identical except for a 61 bp region derived from alternate splicing in *Nedd2s*, the antisense expression is likely to affect the levels of both forms of proteins. The mRNA for *Nedd2s* is low but detectable in FDC-P1 cells, however we have failed to see any effects on the survival of FDC-P1 cells transfected with *Nedd2s* (S.K. et al., unpublished data). These observations suggest that Nedd2s is unlikely to play a major role in the regulation of apoptosis in FDC-P1 cells.

Acknowledgments: I thank Andrea Stylianou for technical assistance, Leonie Ashman and Christopher Juttner for encouragement and support, and Anthony Cambareri for help with the figures. This work was supported in part by a grant and a Florey fellowship to the author from the Research Review Committee of the Royal Adelaide Hospital.

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