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Research paper

Enhancement of chemosensitivity toward anticancer drugs by high expression of caspase-1 in NIH 3T3 cells

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It has been well documented that caspase-1 (interleukin-1β-converting enzyme, ICE) and its related cysteine proteinases such as caspase-3 (CPP32, apopain) and caspase-2 (ICH-1_L) play important roles in apoptosis. In the present study, these genes were inserted into an inducible eukaryotic expression vector, pMSG, and transfected into NIH 3T3 mouse fibroblasts. The expression of caspases-1 and -3 was effectively induced by treatment with dexamethasone (Dex). The expression of caspase-2 was elevated in the transfected cells without treatment with Dex but was not further stimulated by Dex. High expression of these proteases alone induced neither apoptosis-like cell death nor any morphological change. However, the expression of caspase-1 but not of caspase-2 or -3 enhanced chemosensitivity toward cytotoxic anticancer drugs such as aclarubicin, epirubicin, adriamycin, nimustine and ifosfamide. It is thus concluded that caspase-1 mediates cytotoxic effects of these drugs. [© 1998 Rapid Science Ltd.]

Key words: Anticancer drug, apoptosis, caspase.

Introduction

Aspartic acid-specific cysteine proteinases named the caspase family are regarded to play key roles in an apoptotic process based on the following observations. (i) Overexpression of caspases-1 (interleukin-1 β-converting enzyme, ICE), -2 (ICH1), -4 ($ICE_{rel}II$) and -5 ($ICE_{rel}III$) resulted in apoptosis in fibroblast cells.[1–4] (ii) Specific inhibitors of caspases suppressed anti-Fas- and tumor necrosis factor-induced apoptosis.[5–7] (iii) Decreased apoptosis in brain in caspase-3 (CPP32)-deficient mice.[8] (iv) Degradation by caspases of apoptosis-specific substrates such as poly(ADP-ribose) polymerase,[5] Gas2,[9] protein kinase δ,[10] actin,[11] retinoblastoma protein,[12] lamin,[13] fodrin,[14] DNA-dependent protein kinase,[15] etc. Thus far, 10 members of the caspase family have been already reported[16] and it is still obscure how these caspases function.

Apoptosis-like cell death is frequently observed after treatment with cytotoxic anticancer drugs such as cisplatin, doxorubicin, camptothecin, 5-fluorouracil, etc.[17] It is plausible that the caspase family also plays a crucial role in this type of apoptotic process. In the present study, attempts were made to investigate whether the high expression of one of caspases-1, -2 and -3 is sufficient to induce apoptosis, and which caspase mediates cytotoxic drug-induced apoptosis in NIH 3T3 mouse fibroblasts. The results showed that caspase-1 but not -2 or -3 is involved in the cytotoxic effects of anticancer drugs such as adriamycin derivatives.

Materials and methods

Materials

Nimustine [1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-(2-chloroethyl)-3-nitrosourea hydrochloride, ACNU] was obtained from Sankyo; aclarubicin (aclacinomycin-A, ACR), from Yamnouchi Pharmaceutical; and Adriamycin (doxorubicin, ADM), epirubicin (4′epidoxorubicin, 4′epi-Adriamycin, EPI), ifosfamide (IFM), from Shionogi Pharmaceutical. A23187 and MTT (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bro-

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mide) were purchased from Sigma (St Louis, MO). ONO-3403 (ethyl *N*-allyl-N/E-[(E)-2-methyl-3-[4-(4-amidinophenoxycarbonyl)-phenyl]propenoyl]amino acetate methanesulfonate)[18] was provided by ONO Pharmaceutical (Osaka, Japan). ALLN (calpain inhibitor I)[19] was purchased from Nakarai Chemical (Kyoto, Japan), and staurosporine and dexamethasone were from Wako Chemical.

Anti-ICE antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-CPP32 and anti-ICH-1_L antibodies were from Transduction Laboratories (Lexington, KY).

Cell culture

NIH 3T3 mouse fibroblasts and the transfected clones were cultured in Dulbecco's-modified medium supplemented with 10% calf serum.

Construction of plasmids

cDNA clones for human caspases-1, -2 and -3 were provided by Dr Junying Yuan (Massachusetts General Hospital).[1,2] These were inserted into a *Sma*I site of an inducible eukaryotic expression vector, pMSG (Pharmacia). Using this vector, the inserted genes were effectively induced by treatment of the transfected cells with a glucocorticoid hormone, Dex.[20]

Transfection

Caspase-1 (ICE), -3 (CPP32) and -2 (ICH1) genes were co-transfected with the *neo* gene into NIH 3T3 cells using Lipofectin™ (Gibco/BRL). Transfected clones were selected in the presence of G418 (400 μg/ml). NVE, NIC, NCP and NCH represent the transfectants of a vector, caspases-1, -3 and -2 genes, respectively.

Preparation of cell extract

Cells were treated with Dex (1 μM) or the solvent dimethylsulfoxide (DMSO, 0.1%) for 2 days, and then washed with phosphate-buffered saline (PBS) four times and incubated in 0.5% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsufloxide, 10 μM leupeptin, 10 μM pepstatin A and 10 μM ALLN for 10 min at 4°C as described.[21] The cell lysate was centrifuged at 13 000 *g* for 10 min and the supernatant was analyzed by Western blot using the ECL system (Amersham).

MTT assay

Transfected cells were pretreated with DMSO (0.1%) or Dex (1 μM) for 2 days. Then, the cells were replated into 96-well dishes containing various concentrations of test drugs in the presence of DMSO or Dex. After culture for 3 days, the viable cells were estimated by the MTT method of Mosmann[22] as described.[23] Relative viability was shown as a percentage of that in the absence of test drugs.

Results

Expression of caspases in transfectants

It was possible to artificially induce the expression of a gene inserted in a eukaryotic expression vector, pMSG, by treatment of transfected cells with a glucocorticoid hormone, Dex. Figure 1(A) shows the expression levels of caspase-1 (ICE) in the transfected clones. NVE-1 is a control clone transfected with a

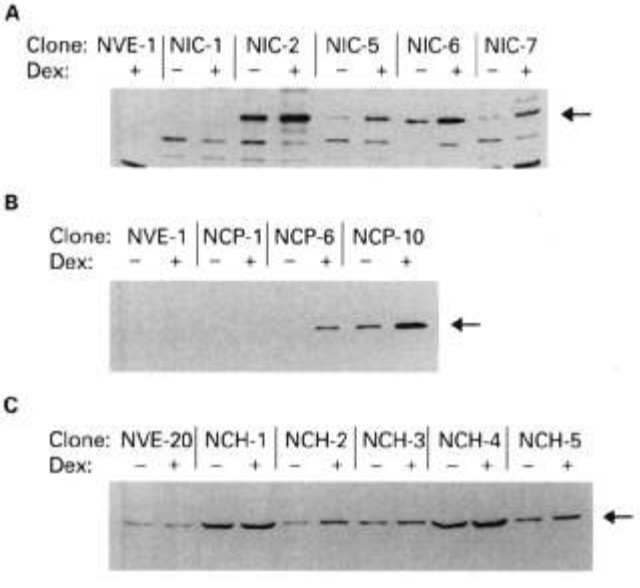


Figure 1. Expression of caspases in transfected cells. NIH 3T3 cells were transfected with a vector or respective caspase cDNA and G418-resistant clones were selected. Cells were treated with (+) or without (−) Dex for 2 days and the cell extracts were analyzed by Western blot. (A) Cell extracts of vector- (NVE-1) or caspase-1-transfected clones (NIC-1, -2, -5, -6 and -7) were analyzed by immunoblot using anti-ICE antibody. (B) Extracts of vector- (NVE-1) or caspase-3-transfected clones (NCP-1, -6 and -10) were analyzed using anti-CPP32 antibody. (C) Extracts of vector- (NVE-20) or caspase-2-transfected clones (NCH-1, -2, -3, -4 and -5) were analyzed using anti-ICH-1 antibody. Arrows indicate the positions of each caspase.

vector gene alone and no significant reaction of endogenous caspase-1 was observed. Among caspase-1-transfected clones, NIC-2 and NIC-6 showed a large increase in the expression of caspase-1 after treatment with Dex. However, a moderate level of caspase-1 expression was also observed without treatment with Dex, possibly due to the leakiness of this expression vector as discussed previously.[20] Very low or no expression of caspase-1 was detected in NIC-1, -5 and -7 even after treatment with Dex.

Similar expression was also observed in caspase-3-transfected clones (Figure 1B). NCP-10 showed high expression of caspase-3 and a small amount of caspase-3 was detectable in NCP-6 only after treatment with Dex. However, no significant expression was observed in NCP-1 as well as in a vector control clone, NVE-1.

A low level of caspase-2 expression was observed in a vector-transfected NVE-20 (Figure 1C). This level may reflect the endogenous amount of caspase-2. Among the transfected clones, NCH-1 and -4 showed the elevated expression of caspase-2. However, this expression was unaffected by treatment with Dex. Our preliminary results showed that glucocorticoid receptors were down-regulated in caspase-2-transfected clones, suggesing that the increase in caspase-2 expression in NCH-1 and -4 was indpendent of glucocortcoid receptors but due to the unidentified leaked expression of the expression vector.

Differential chemosensitivity of caspase-transfected clones

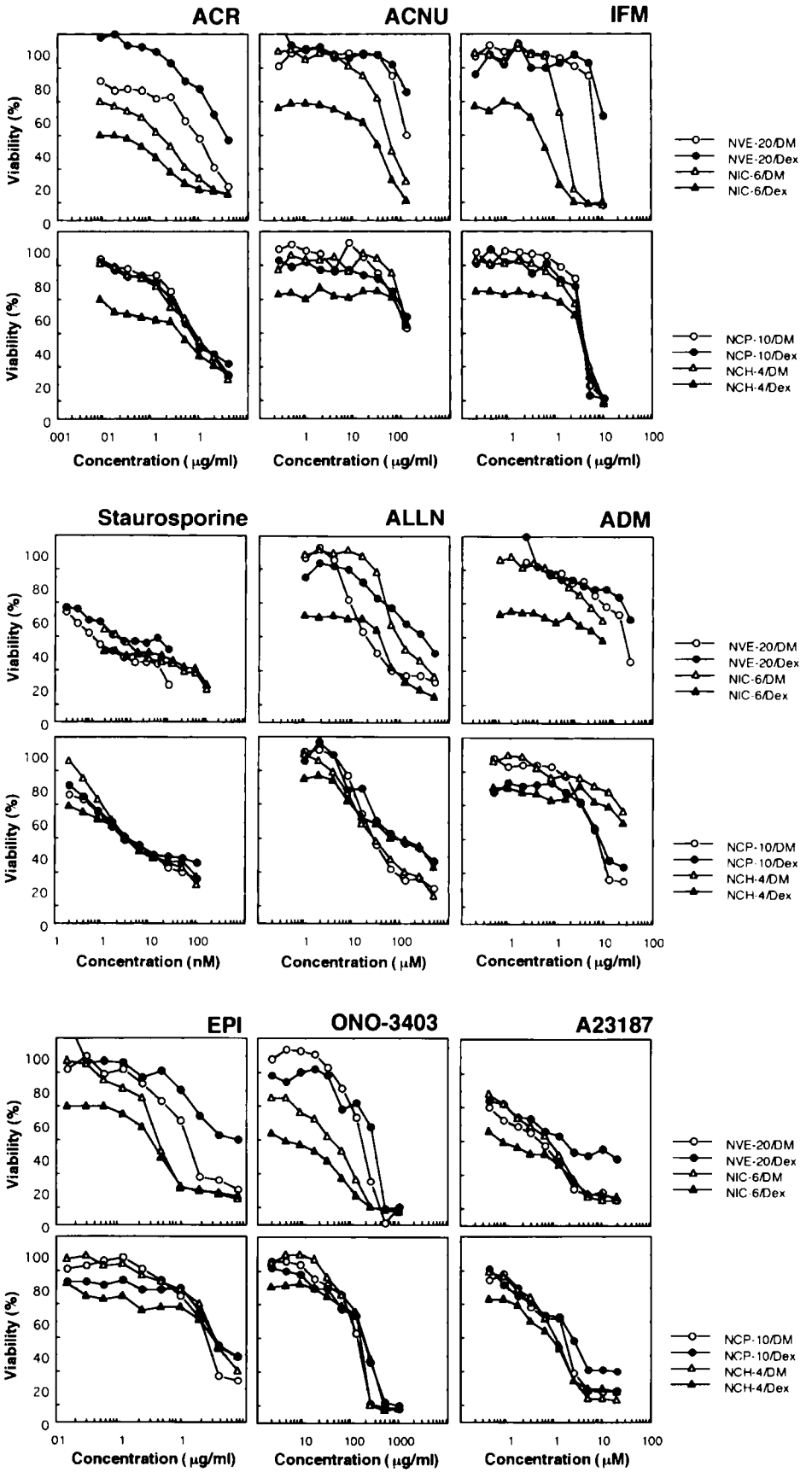
It has been reported that the high expression of caspase-1 or -2 alone is sufficient to induce apoptosis-like cell death of Rat-1 and HeLa cells.[1,2] However, in spite of the aforementioned high expression of transfected genes in NIH 3T3 cells, none of the transfectants showed apoptosis-like alterations. For example, phase morphology of NIC-6, NCP-10 and NCH-4 was almost the same as that of the parent NIH 3T3 or vector-transfected clones and irrespective of the treatment with Dex (data not shown). Even when apoptotic cells were investigated by DNA fragmentation analysis or by TUNEL staining,[24] no significant evidence was obtained (data not shown). These results suggest that the high expression of caspase-1, -2 or -3 alone is not sufficient to induce apoptosis in NIH 3T3 cells, which is different from Rat-1 and HeLa cells.

However, even if the simple overexpression of a caspase is insufficient for induction of apoptosis, such high expression might influence the apoptotic process triggered by various agents. Thus, the transfected clones were treated with various cytotoxic drugs in the presence or absence of Dex. After treatment for 2 days, the viable cells were estimated by the MTT method (Figure 2). The viability was dose dependently decreased by cytotoxic drugs. A high producer of caspase-1 (NIC-6) showed higher sensitivity toward cytotoxic anticancer drugs such as ACR, EPI, ADM, ACNU and IFM as compared to those of vector-, caspase-3- and caspase-2-transfected clones (NVE-20, NCP-10 and NCH-4, respectively). The sensitivity of NIC-6 was even more prominent in the presence of Dex which increased caspase-1 expression (Figure 1A). Another high producer of caspase-1, NIC-2, also showed similar results (data not shown). It is thus at least evident that caspase-1 mediates the cytotoxic effects of some anticancer drugs such as ACR, EPI, ADM, ACNU and IFM.

On the other hand, caspase-2-transfected clone, NCH-4, exhibited the slightly higher sensitivity toward ACR, ACNU and IFM only in the presence of Dex than those of vector- and caspase-3-transfected clones. Despite the effective induction of the expression of caspase-3 by treatment with Dex, the chemosensitivity of caspase-3-producing cells toward test drugs was comparable to that of the vector control under the present experimental conditions. These results do not support the previous report that caspase-3 plays a crucial role in apoptosis.[7,8]

It is well known that a calcium ionophore, A23187, and a protein kinase inhibitor, staurosporine, are apoptosis inducers.[25,26] ONO-3403 and ALLN, which inhibit serine proteases and proteasome, respectively, also induce apoptosis.[27,28] Cytotoxic growth-inhibitory activities of these compounds were not significantly affected by the high expression of caspases-1, -2 and -3 except synergistic growth suppression by ONO-3403 and caspase-1 (Figure 2).

Figure 2. Chemosensitivity of NIH 3T3 transfected cells toward chemical carcinogens, A23187, ONO-3403, staurosporine and ALLN. Transfected cells were cultured for 3 days in the presence of varying concentrations of test drugs and Dex (1 μM) (closed symbols) or a solvent DMSO (0.1%) (open symbols). Viable cells were measured by the MTT method.[22] The upper panels indicate the results of vector- (NVE-20; circles) and caspase-1-transfected (NIC-6; triangles) clones. The lower panels indicate the results of caspase-3- (NCP-10; circles) and caspase-2-transfected (NCH-4; triangles) clones. The ordinate and the abscissa represent relative viability (%) and the concentration of each test drug, respectively.


ACR
ACNU
IFM
Staurosporine
ALLN
ADM
EPI
ONO-3403
A23187
Viability (%)
Concentration (μg/ml)
Concentration (nM)
Concentration (μM)
NVE-20/DM
NVE-20/Dex
NIC-6/DM
NIC-6/Dex
NCP-10/DM
NCP-10/Dex
NCH-4/DM
NCH-4/Dex

Discussion

Caspase-1

We have attained an induction of high expression of caspases-1, -2 and -3 in NIH 3T3 mouse fibroblasts. However, none of the signs of apoptosis-like cell death were observed even after the induction. This is inconsistent with previous reports that the expression of one of these caspases alone is sufficient to produce apoptosis of Rat-1 and HeLa cells.[1-3] This discrepancy might be due to cell types. Rat-1 could be an apoptosis-competent cell and has frequently been used in apoptosis research. Increased expression of only one candidate might trigger apoptosis in this cell line. On the other hand, NIH 3T3 cells are very resistant to apoptosis inducers.

Enhanced sensitivity in a caspase-1-transfected clone (NIC-6) was observed toward ACR, EPI, ADM, ACNU and IFM. It is noteworthy that ACR, EPI and ADM have similar chemical structures and showed high growth-inhibitory activities toward rapidly growing murine transformed cells.[29] ACNU and IFM are alkylating agents and possess similar growth-inhibitory activities toward murine fibroblasts.[29] The present results indicate that the cytotoxic effects of these anticancer drugs are mediated by caspase-1. This also suggests the effective application of these anticancer drugs for clinical use, i.e. the effective chemotherapy by using these drugs could be expected only when they are applied to caspase-1-producing tumor cells.

Chemosensitivity to A23187, staurosporine and ALLN was not significantly affected by the expression of caspases-1, -2 and -3 (Figure 2). Hence, it is unlikely that these caspases are directly involved in the cytotoxic effects of those drugs. ONO-3403 synergistically inhibited cell growth with caspase-1 (Figure 2). This drug potently inhibits trypsin and some other serine proteases[18] but activates calcium-activated cysteine proteinase, calpain, at the cytotoxic concentrations.[27] It was thus proposed that activation of calpain may account for apoptotic cell death after treatment with ONO-3403. The synergistic growth inhibition by ONO-3403 and caspase-1 suggests a possible relation between caspase-1 and calpain.

Caspase-2

Our present results showed that the high expression of human caspase-2 (ICH-1) alone did not induce cell death of mouse NIH 3T3 cells. A murine homolog of human caspase-2 is *Nedd2* which induced apoptosis in NIH 3T3 cells.[3] It is thus possible that species specificity might have some restriction toward the activity of caspase-2 in murine cells.

We could not obtain a transfectant which showed a large increase in the expression of caspase-2 after treatment with Dex. This suggests that caspase-2 might disturb glucocorticoid hormone receptor-mediated signal transduction and restrict the gene expression regulated by the receptor. It should be noted that glucocorticoid induces apoptosis although the molecular mechanisms are poorly understood.[30] Therefore, it is possible that caspase-2 is involved in glucocorticoid-induced apoptosis. We cannot completely rule out the possibility that an increase in caspase-2 expression results in cell killing and hence loss of the killed cells from the analyzed population.

Caspase-3

A number of reports have shown that caspase-3 (CPP32) has a key role in induction of apoptosis.[7,8,32] This appears to be incompatible with our present results. However, some explanations could be available to account for this discrepancy. (i) Caspase-3 inhibitors such as CrmA and DEVD-CHO were frequently used to discriminate each caspase.[5,32] However, there are other caspase-3-like proteases such as Mch2 and Mch3 (caspases-6 and -7, respectively). Thus, it is very difficult to determine the types of caspases based on the results of inhibitors. It was also reported that activation of caspase-3 alone did not induce apoptosis in T lymphocyte.[32] Accordingly, the function of caspase-3 might be replaced by those of other caspases in NIH 3T3 cells. (ii) Although the endogenous expression level of caspase-2 was undetectable in NIH 3T3 cells, it is still possible that a very low level of the caspase might be sufficient to mediate the apoptotic signaling, i.e. the expression level of caspase-3 is not limiting in induction of apoptosis.

Perspective

It has been shown in the present study that caspase-1 mediates the cytotoxic effects of anticancer drugs. However, the specific functions of other caspases remain to be determined. Is each caspase required for activation of the subsequent caspase in the protease cascade? Alternatively, can apoptosis-associated substrates be cleaved by most, if not all, of the caspases non-specifically? It is also enigmatic how the caspase family can interact with other apoptosis-relating molecules such as the Bcl-2 family, p53, NFκB, etc. The answers to these questions will facilitate the clinical application of cytotoxic drugs for chemotherapy.

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