Modulation of c-JUN expression in leukemic cells undergoing apoptosis after chemotherapeutic treatment

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Anticancer treatments are supposed to kill cancer cells by activating the key elements of the apoptosis program. Diverse upstream signaling pathways converge and activate the core components of cell death machinery. Transcription factor encoding protein c-Jun provides a useful model for studying the complexity and specificity of apoptotic signaling. c-Jun is an inducible transcription factor which directs changes of gene expression in response to multiple extracellular stimuli.

The present study was designed to determine changes of c-Jun protein expression during apoptosis of hematopoietic tumor cells after chemotherapeutic treatments. Apoptosis was induced in human leukemic HL-60, HEL and K562 cells with drugs of different intracellular targets. Here we report that all the cytotoxic drugs tested induced c-Jun augmentation as well as phosphorylation, although with different kinetics. The increase of c-Jun expression in response to cytotoxic agents paralelled with induction of apoptosis. Our results demonstrated for the first time that a wide variety of apoptotic stimuli induced c-Jun cleavage in human hematopoietic tumor cells of different origin.

Key words: apoptosis, leukemic cells, chemotherapeutic drugs, c-Jun expression, cleavage

INTRODUCTION

With identification and characterization of signaling mechanisms that govern cell growth, differentiation, motility and apoptosis, the transition from oncogenomics to mechanism based cancer medicine has begun.

Green and Evan have suggested that deregulation of proliferation, together with reduction in apoptosis are both necessary and can be sufficient for cancer [1]. Apoptosis is a multi-step, multi-pathway physiological cell-death process of eliminating unwanted cells from living organisms during embryonic and adult development. Tumour cells can acquire resistance to apoptosis by the expression of antiapoptotic proteins or by the down regulation or mutation of proapoptotic proteins [2]. Induction of apoptosis in cancer cells is the mode of action of many anti-tumour agents. Anticancer treatments are supposed to kill target cells by activating the key elements of the apoptosis program. Understanding the molecular mechanism of drug-induced apoptosis as well as the alterations in cancer cells which render cancer cells resistant to apoptotic treatments provides the new molecular targets for therapy in cancer. Modulation of the key elements of apoptosis signaling systems directly modifies therapy-induced tumour-cell death [3, 4].

Diverse upstream signaling pathways converge and activate the core components of cell death machinery. Among the best-characterized pathways regulating cell survival and cell death are members of the mitogen-activated protein kinase (MAPK) family. The widespread involvement of MAP kinase cascades in death and survival signaling makes them potentially useful for therapeutic modulation. Although the physiological consequences of MAPK activation is cell-type specific, evidence indicates that the extracellular signal regulated kinase (ERK) is generally involved in control of cell proliferation and may counteract apoptotic signaling. In contrast, ac-

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tivation of the c-Jun N-terminal kinase/stress activated kinase (JNK/SAPK) and p38 or HOG MAP kinase pathways has been suggested to be critical for induction of apoptosis in some apoptotic systems [5, 6]. Although the exact mechanism by which the JNK and p38 MAP kinase pathways induce apoptosis remains unknown, their key role as modulators of transcription factor activity is important.

The transcription factor c-Jun provides the complexity and specificity of signaling. c-Jun is an inducible transcription factor, which directs changes of gene expression in response of multiple extracellular stimuli. c-Jun is, together with the Fos family of proteins, a major component of the AP-1 transcriptional complex. Jun-related proteins can funcion either as homodimer or as heterodimers bound to partner proteins such as Fos-related proteins, CREB or ATF2. The AP-1 transcription complex has been implicated in a number of biological processes such as proliferation, differentiation, transformation, cellular stress response. AP-1 activity is controlled primarily by the abundance and phosphorylation status of these proteins. The proto-oncogene *c-jun*, together with other members of this gene family is rapidly induced by mitogenic agents and is refered as the early response gene. In addition to physiological stimuli transactivation of *c-jun* mRNA rises after exposure of cell to various forms of stress. The *c-jun* proto-oncogene is positively autoregulated by its own gene product. In addition, regulation of c-Jun activity can also be regulated directly at protein level. Two MAPK cascades converge on c-Jun. The activation of ERK pathway in PC12 cells results in stimulation of both c-Jun synthesis and c--Jun phosphorylation, whereas the JNK pathway triggers phosphorylation only. c-Jun is the best-characterized substrate of the proapoptotic MAP kinase JNK. c-Jun is phosphorylated at Ser 63, Ser 73, Thr 91 and/or Thr 93 within the N-terminal portion of the molecule by MAP kinases of the ERK and JNK family. Phosphorylation of these sites induces an increase in the DNA binding and transactivation potential of protein as well as an increase in the stability of c-Jun [7–9].

In the present study we examined the expression of c-Jun in the apoptosis in human leukemic cells induced by different settings of chemotherapeutic agents. Induction of c-Jun expression and phosphorylation as well as c-Jun protein cleavage were detected in human hematopoietic cells undergoing apoptosis in response to a variety of different treatments.

MATHERIALS AND METHODS

Cell culture and treatments. Human hematopoietic tumour line HL-60, HEL and K562 cells were cultured in RPMI medium supplemented with 10% of fetal bovine serum with antibiotics. Exponentially growing cells were seeded at 5×10^5/ml and treated with chemotherapeutic agents for various periods of time. All chemicals used for apoptotic treatments were purchased from Sigma-Aldrich (St. Louis, MO). Dr. K. Ollinger, Linkoping University, kindly provided naphthazarin (Nz). Stock solutions of naphthazarin and camptothecin were prepared in DMSO. Both cisplatin (cis-platinum (II) diamine dichloride) and colchicine were dissolved in saline as 10 mg/ml stock solutions. Cells were treated with a final concentration 0.5 μM of naphthazarin, 1 μg/ml of colchicin, 1 μM of camptothecin, 10 μg/ml of cisplatin in a complete medium.

Analysis of apoptotic cell death. Apoptosis was evaluated using the acridine orange/ ethidium bromide (AO/AB) staining technique with fluorescence microscopy [10]. Cells belonging to the following groups were counted: 1) viable cells with non-apoptotic nuclei (VNA, bright-green chromatin with an organised structure); 2) viable cells with apoptotic nuclei (VA, bright green chromatin that is highly condensed or fragmented); 3) nonviable apoptotic cells (NVA, bright-orange chromatin that is highly condensed or fragmented); 4) necrotic cells (NEC, bright-orange chromatin with an organised structure); 5) chromatin-free cells (CF, cells that have totally lost their DNA content and are exhibiting a weak green-orange staining).

Western blot analysis. After an appropriate treatment cells were collected, washed in ice-cold PBS and lysed (on ice) with a lysis buffer (10 mM TrisHCl, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 20 μg/ml aprotinin, 1 mM PMSF, 2 mM Na_3VO_4). The lysates were centrifuged for 15 min, 15000 rpm at 4 °C. The different extracts were resolved on 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes and blocked in Blotto. Membranes were probed with monoclonal anti-c-Jun primary antibodies (Transduction Laboratories) followed by horseradish peroxidase-conjugated secondary antibodies. Proteins were detected using the enhanced chemiluminescence (ECL) method according to manufacturer's instructions (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

In this study, we examined the level of apoptosis and changes of c-Jun expression in different human leukemia cell lines in response to diverse classes of antitumor agents. Chemotherapeutic agents with different intracellular molecular targets were used. Apoptosis was induced in leukemic cells by the redox-cycling quinone naphthazarin (5,8-dihydroxy-1,4-

naphthoquinone), microtubule disruptor colchicin, topoizomerase I inhibitor camptothecin, and the DNA damaging agent cisplatin, inducing inter- and intrastrand cross links. The chemotherapeutic agents induced apoptosis in cells tested with different kinetics and intensity (Fig. 1).

The results obtained show that human promyelocytic leukemia HL-60 cells are highly sensitive to cytotoxic treatments. A very potent inducer of apoptosis in HL-60 cells was naphthazarin (Nz). The highest amount of apoptotic cells was reached in 12 hours (Fig. 1, A). The kinetics of appearance of apoptotic HL-60 cells was slower after colchicin and camptothecin treatment: data presented in Fig. 1 show that after 24 h of treatment apoptotic cells in the population made up 34 and 51 per cent, respectively. Less efficiently cytotoxic drugs induced apoptosis in human erythroleukemia HEL cells. K562 chronic myelogenous leukemia, a p210 BCR-ABL transformed cell line, cells were the most resistant to all apoptotic treatment (data not presented). Figure 1 (B, C) shows apoptotic response of HEL and K562 cells to anticancer drug camptothecin and cisplatin treatment.

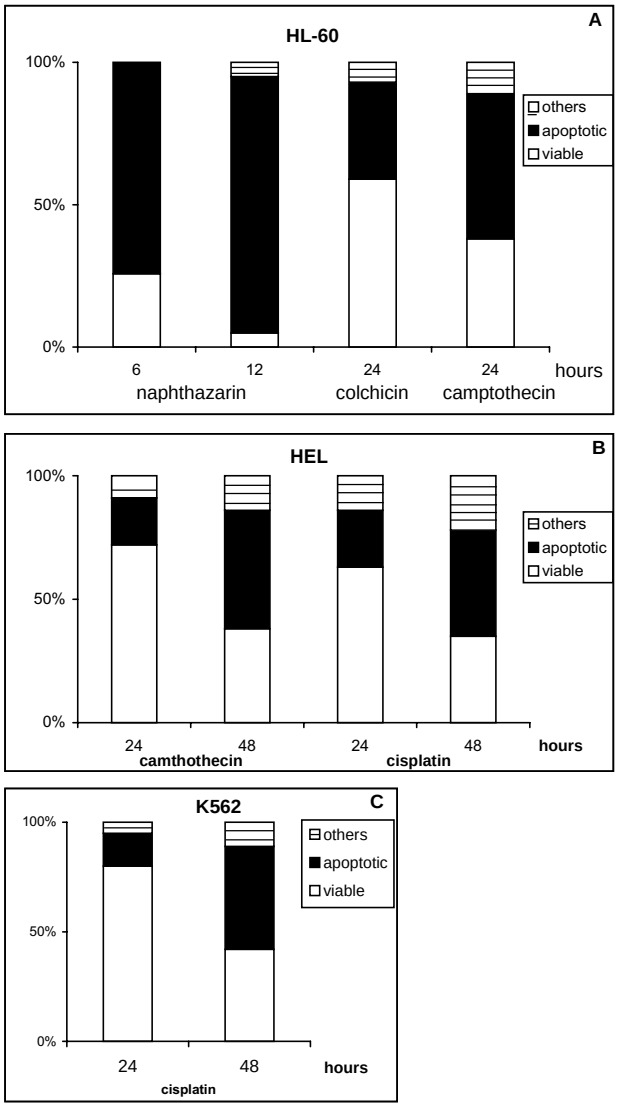

Fig. 1. Apoptotic response of leukemic cells to chemotherapeutic drugs. The percentages of apoptotic cells were quantitated using the AO/EB staining technique at different time-points. Cells were treated with 0.5 μM of naphthazarin, 1 μg/ml of colchicin, 0.5 – 1 μM of camptothecin, 10 μg/ml of cisplatin in complete medium for the time indicated

Studies of c-Jun expression were performed in leukemic cells undergoing apoptosis after treatment with cytotoxic drugs possessing different intracellular targets. Although leukemic cells differed in respect of the basal c-Jun protein level (K562 cells had the highest content of c-Jun protein), time course experiments revealed a time-dependent increase of the level of c-Jun protein in all induced cells (Figs. 2, 3). The intensity of the band of c-Jun increased gradually after all treatments. The pattern of c-Jun expression varied with the inducing stimulus and this paralleled with apoptotic changes. In addition, a mobility shifted phosphorylated form of c-Jun was observed (Fig. 2, 3, upper arrows). Induction of c-Jun phosphorylation coincided with augmentation of its expression.

Moreover, the results obtained indicate that c-Jun is cleaved in cytotoxic-drug-induced apoptosis in leukemic cells (Figs. 2, 3, lower arrows). Appearance of about 30 kDa c-Jun fragments was noticeable at the

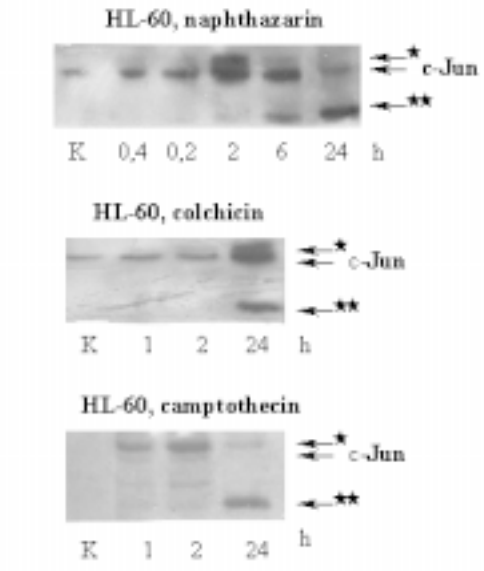

Fig. 2. Time-dependent effects of chemotherapeutic treatments on c-Jun protein in HL-60 cells in culture. Cells were treated as in Fig. 1 for the time indicated. Whole cell lysates were prepared and analysed by using anti-c-Jun antibodies as described in Materials and methods. Upper and lower arrows indicate electrophoretic retardation of phosphorylated form of c-Jun (*) and c-Jun cleavage (**), respectively

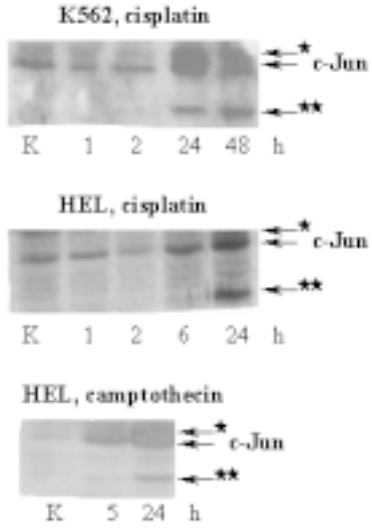

Fig. 3. Time-dependent effects of DNA damaging anticancer drugs camptothecin and cisplatin on c-Jun protein in HEL and K562 cells in culture. Cells were treated as in Fig. 1 for the time indicated. Upper and lower arrows indicate electrophoretic retardation of phosphorylated form of c-Jun (*) and c-Jun cleavage (**), respectively

time of arising of apoptotic cells. The data indicate that this cleavage is caspase-dependent (data not presented). As far as the authors know, this is the first evidence of the caspase-dependent cleavage of c-Jun protein in cells undergoing apoptosis. Signalling proteins as substrates for proteolysis during apoptosis are considered as positive or negative regulators of apoptosis. Cleavage of Ras, GAP, Raf, Cbl, Akt-1, *etc.* in response to apoptotic stimuli has been demonstrated [11].

In conclusion the current results indicate an association between the c-Jun expression, its modification, and induction of apoptosis in response to anticancer drugs. Different lines of evidence suggest the role of c-Jun as the mediator of apoptosis. Activation of c-Jun regulates apoptosis through modulation of certain proteins required in the apoptotic response or through sequestration of inhibitors of apoptosis. Cell death induced by overexpression of *c-jun* depends upon the amino-terminal region containing a transactivation region that includes the leucin zipper domain necessary for dimerization [12, 13]. On the other hand, there is evidence that induction of c-Jun expression has no direct role in the drug-induced apoptosis and that apoptosis can occur by the mechanisms that do not involve induction of c-Jun expression [14]. Our preliminary results using *c-jun* antisense show that c-Jun protein is relevant to hematopoietic cancer cell apoptosis.

Our results have demonstrated for the first time that c-Jun is subjected to cleavage during chemotherapeutic agent-induced apoptosis. Further studies to evaluate the role of this cleavage are in progress.

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References

1. Green DG, Evan GI. Cancer cell 2002; 1: 19–30.
2. Haq R, Zanke B. Cancer and Metastasis 1998; 17: 233–9.
3. Makin G, Dive C. Trends Cell Biol 2001; 11: S22–6.
4. Huang P, Oliff A. Trends Cell Biol 2001; 11: 343–8.
5. Franklin RA, Cubrey JA. Leukemia 2000; 14: 2019–34.
6. Xia Z, Dickens M, Raingeaud J et al. Science 1995; 270: 1326–31.
7. Vogt PK. Oncogene 2001; 20: 2365–77.
8. Leppa S, Saffrich R, Ansorg W et al. The EMBO Journal 1998; 17: 4404–13.
9. Chen Y, Tan T. International Journal of Oncology 2000; 16: 651–62.
10. Mercille S, Massie B. Biotechn Bioeng 1994; 44: 1140–53.
11. Widmann C, Gibson S, Johnson GL. J Biol Chem 1998; 273 (12): 7141–7.
12. Bossy-Wetzel E, Bakiri L, Yaniv M. The EMBO Journal 1997; 16 (7): 1695–1709.
13. Kolbus A, Herr I, Schreiber M et al. Mol Cell Biol 2000; 20(2): 575–82.
14. Shaulian E, Karin M. Oncogene 2001; 20: 2390–400.
15. Galan A, Garcia-Bermejo L, Vilaboa NE et al. Eur J Cell Biol 2000; 79 (1): 1–9.

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C-JUN RAIŠKOS MODULIACIJA LEUKEMINĖSE LĄSTELĖSE CHEMOTERAPINIŲ MEDŽIAGŲ INDUKUOTOS APOPTOZĖS METU

S a n t r a u k a

Manoma, kad priešvėžiniai vaistai indukuoja užprogramuotą ląstelės žūtį (apoptozę) aktyvuodami ar moduliuodami apoptozės signalinių kelių komponentus. Vienas iš galimų apoptozės reguliatorių – c-JUN baltymas, AP-1 transkripcijos komplekso komponentas, aktyvuotų signalinių kaskadų taikinys. Tyrėme leukeminių HL-60, HEL, K562 linijų ląstelių apoptozę, indukuotą chemoterapinėmis medžiagomis, turinčiomis skirtingus viduląstelinius taikinius. Skirtingų tipų leukeminės ląstelės buvo nevienodai jautrios apoptozės induktoriams. Nustatyta c-JUN raiškos ir fosforilinimo aktyvacija chemoterapinių medžiagų indukuotos apoptozės metu. Baltymo kiekio didėjimas ir fosforilinimas skyrėsi priklausomai nuo apoptozės induktoriaus ir ląstelių tipo, bet koreliavo su apoptozės indukcija.

Pirmą kartą nustatytas nuo kaspazių priklausomas c-JUN baltymo skaidymas ląstelėse apoptozės metu.