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Caspase 3, periodically expressed and activated at G2/M transition, is required for nocodazole-induced mitotic checkpoint

S.-L. Hsu*, C.-T. R. Yu*, S.-C. Yin*, M.-J. Tang *, A.-C. Tien, Y.-M. Wu and C.-Y. F. Huang

Department of Education and Research, Taichung Veterans General Hospital, Taichung 407, Taiwan (S.-L. Hsu*, S.-C. Yin*); Institute of Public Health School of Medicine, National Yang-Ming University, Taipei 112, Taiwan R.O.C (C.-T. R. Yu*); Graduate Institute of Clinical Medicine (M.-J. Tang *); and Department of Physiology, National Cheng Kung University Medical College, Tainan 701, Taiwan (M.-J. Tang *); National Cancer Research Center, National Health Research Institutes, Taipei 115, Taiwan (A.-C. Tien, Y.-M. Wu, C.-Y. F. Huang); Graduate Institute of Life Sciences, National Defense Medical Center, Taipei 114, Taiwan R.O.C. (C.-Y. F. Huang); Institute of Biotechnology in Medicine, National Yang-Ming University, Taipei 112, Taiwan R.O.C. (C.-Y. F. Huang)

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Caspases have been known for several years for their involvement in executing apoptosis, where unwanted or damaged cells are eliminated. Surprisingly, after analysis of the relevant data set from the Stanford microarray database, we noticed that the gene expression pattern for caspase 3, but not for caspase 1, 6, 7, 8, 9, or 10, undergoes periodic change in the HeLa cell cycle. In this study, we have demonstrated that caspase 3, but not other caspases, is upregulated and activated just prior to mitosis. Pretreatment of human hepatoma cells with a caspase 3 inhibitor z-DEVD-FMK, prior to the treatment with an antimicrotubule drug nocodazole, abrogates the mitotic arrest, suggesting that caspase 3 (or a caspase 3-like enzyme) might be involved in mitotic-spindle checkpoint. The studies not only characterize caspase 3 as a cell cycle-regulated protein, but also link the protein to nocodazole-dependent mitotic checkpoint, greatly expanding the understanding of caspase 3.

Key words: caspase 3; mitotic checkpoint; nocodazole.

Introduction

Apoptosis is a complex biological process that enables an organism to eliminate unwanted or defective cells through an orderly process of cellular disintegration.[1–3]

Although the apoptotic stimuli that elicit responses vary from cell to cell, there seems to be a basic biochemical machinery underlying the process of programmed cell death. Growing evidence suggests that apoptosis is mediated by the activation of a family of cysteine proteases, known as caspases.[4–6] To date, 14 caspases have been identified.[6] All of them share similarities in the primary sequence and 3-dimentional structure. In addition, these caspases are all expressed as proenzymes activated by proteolytic processing followed by dimerization. In response to pro-apoptotic signals, caspases act, in part, by activating their downstream analogs (*e.g.*, caspase 8 activates the downstream effector caspase 3) and, in part, by cleaving a set of proteins, resulting in an inactivation of the proteins necessary for cell survival, structural integrity or apoptosis inhibition. To date, a large number of caspase substrates have been identified.[7–10] However, the functional significance of the majority of these cleavage events, and their exact roles in the execution of apoptosis, and possible other functions are not yet fully understood.

In addition to their role in apoptosis, the caspases may also have other functions for the study. For example, recent studies have shown that caspase 3 activity is required for skeletal muscle differentiation.[11] Caspase activation is also required for T cell proliferation,[12] and that activated caspases can cleave selective substrates in non-apoptotic cells during T lymphocyte stimulation.[13] Furthermore, the application of microarray profiling has produced major opportunities for the elaboration of the cell-signature changes that occur, for example, during the cell cycle. Of the periodically expressed genes, the gene-expression profile for caspase 3 has been assigned to the group of cell cycle-regulated genes.[14] However, the role of caspase 3 in the cell cycle has not been explored. In the current study, we have demonstrated that caspase 3 (or a caspase 3-like enzyme) is up regulated and activated immediately before cells enter mitosis. The action of nocodazole, an antimicrotubule drug, in provoking a mitotic arrest is attenuated in the presence of caspase 3 inhibitor z-DEVD-FMK, supporting the notion that caspase 3 and/or some subset of caspase 3 substrate(s) may have a unique function in the nocodazole-dependent mitotic checkpoint.

*These authors contributed equally.

Correspondence to: C.-Y. F. Huang, National Cancer Research Center, National Health Research Institutes, 9F. Room 9323, No. 161, Sec. 6, Min-Chuan East Road, Taipei 114, Taiwan, R.O.C. e-mail: chiying@nhri.org.tw

Materials and methods

Cell culture, cell cycle synchronization and cell cycle analysis by flow cytometry

HeLa, HepG2 and Hep3B cells, from the American Type Culture Collection, maintained at 37°C in a 5% CO_2 incubator, were grown in DMEM or RPMI medium supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/ml penicillin/streptomycin (BRL). The HeLa cells were synchronized at the G1/S boundary using thymidine and aphidicolin. Briefly, cells were cultured for 14 h in the presence of 2 mM thymidine (Sigma), released for 12 h in fresh medium, and finally arrested for 14 h in the presence of 1.6 μg/ml aphidicolin (Sigma). Cells were released from the aphidicolin block by changing them to fresh drug-free culture medium. The percentage of cells in different phases of the cell cycle at each time point was quantified using flow cytometry analysis (Becton Dickinson FACStar Plus), and the mitotic cells were scored by mitotic index examination. Briefly, 1×10^6 cells were trypsinized, washed with PBS, and fixed in 80% ethanol, then washed with PBS, incubated with 100 μg/ml RNase at 37°C for 30 min, stained with propidium iodide (50 μg/ml), and analyzed on a FACS can flow cytometer. The percentage of cells in different phases including sub-G1 of the cell cycle was analyzed using Cell-FIT software (Becton Dickinson Instruments). The cells with sub-G1 DNA content were considered as apoptotic cells.[17–19] (these are reference numbers). The mitotic index was measured by visualization of cells with DAPI staining under fluorescent microscopy. Approximately, 500–1000 cells were counted at each time point. Cells in mitosis were judged by the appearance of condensed chromosomes.

Western blot

After release from the aphidicolin block, cells were collected at various time intervals, washed twice with PBS and lysed in lysis buffer (20 mM PIPES, pH 7.2, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 1 mM DTT, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, and 10 μg/ml each of leupeptin, aprotinin, chymostatin and pepstatin). After incubation at 4°C for 30 min, cellular debris was removed by centrifugation at 15,000 rpm for 10 min in an Eppendorf centrifuge. Protein concentrations were determined by Bradford assay (BIO-RAD). Total cell lysates were resuspended in SDS sample buffer and resolved using SDS-PAGE. The proteins were transferred to PVDF membranes (Millipore). Subsequently, the membrane was blocked with 5% nonfat milk in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20), and incubated overnight at 4°C with specific primary antibodies, including anti-human caspase 1 (UPSTATE), 3 (Pharmingen), 8 (BD Pharmingen) and 9 (Oncogene) antibodies. Subsequently, the membrane was washed with TBST buffer and incubated with the appropriate secondary antibody (horseradish peroxidase- conjugated goat anti-mouse or anti-rabbit IgG), followed by development of the membranes with enhanced chemiluminescence kits (Amersham; ECL kit).

Caspase activity assay

Caspase activity was measured using specific artificial substrates as described previously.[20] These substrates are able to generate fluorescence when they are catalyzed by specific caspases. The corresponding substrates for caspase 1, 2, 3, 6, 8 and 9 are YVAD-, VDVAD-, DEVD-, VEID-, IETD- and LEHD-AFC (R&D). For checkpoint analysis, Hep3B and HepG2 cells were treated with or without various caspase inhibitors (400 μM) for 8 h and then incubated with nocodazole (50 ng/ml) for 16 h. Cells were then collected for flow cytometry. The inhibitors used to block the various caspases were z-DEVD-FMK, z-IETD-FMK, z-LEHD-FMK and BOC-D-FMK for caspase 3, 8, 9 and pan caspase inhibitor z-VAD-FMK, respectively (KAMIYA Biomedical).

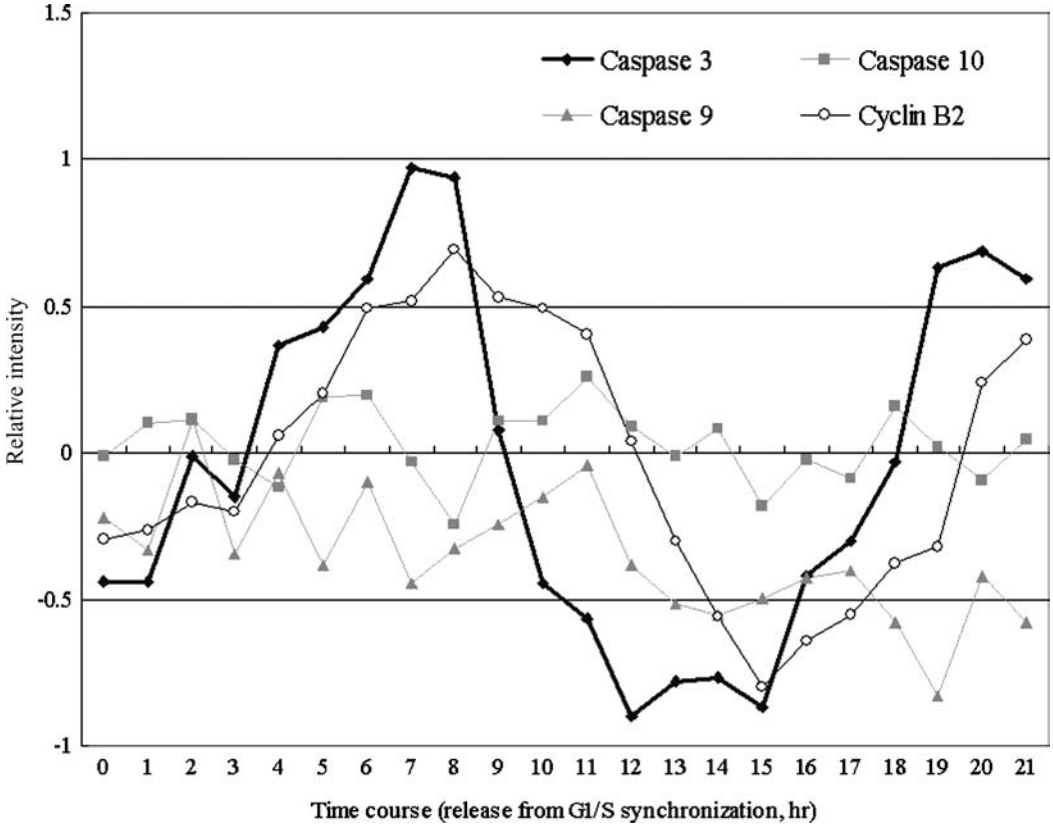
Results and discussion

Overexpression and activation of caspase 3 at the G2/M transition

Genome-wide microarray profiling of human tumors and tumor cell lines has provided invaluable information and proffered unique opportunities inviting further exploration. To search for the interface between apoptosis and cell cycle progression, a series of apoptosis-related markers in the published HeLa cell cycle database (http://genome-www.stanford.edu/Human-CellCycle/HeLa) was systematically analyzed.[14] It was noticed that the gene expression pattern of caspase 3, but not of caspase 1, 6, 7, 8, 9 or 10 (there is no data available for other caspases), undergoes periodic change in the HeLa cell cycle. The expression profiling of the caspases and the mitosis marker, cyclin B2, were downloaded from the above website and replotted (Figure 1). Interestingly, the expression of caspase 3 peaked in the G2 and M phases, slightly ahead of cyclin B2.

To validate the microarray finding and to explore the possible role of caspase 3 in the cell cycle, particularly in the G2/M transition, HeLa cells were synchronized at G1/S using thymidine/aphidicolin block (Figure 2B). Total proteins were then extracted from cells harvested for Western analysis or caspase-activity assay at various times following release from the G1/S block (Figure 2A). FACS analysis (Figure 2B) indicated that cells progressed through the cell cycle with a relatively high degree of synchrony and a very low proportion of apoptotic cells (less than 5% throughout the cell cycle). It is crucial that the number of apoptotic cells

Figure 1. The correlated gene-expression profiles for caspase 3, 9, 10 and cyclin B. The data set was downloaded from the database available at http://genome-www.stanford.edu/Human-CellCycle/HeLa and re-plotted in the current format. The experimental conditions are also detailed on this website. Briefly, HeLa cells were synchronized at G1/S using a double thymidine block. Total RNAs were extracted from cells harvested at various times after the release from G1/S transition and subjected to microarray analysis.[14] Expression of caspase 3 was elevated at 7–8 h, however, cyclin B reached its peak at 8–9 h after G1/S transition.



(sub-G1) remains fairly constant throughout the cell cycle because any sudden stage-specific change in caspase activity could not then be reliably attributed to the drug (thymidine and aphidicolin) treatment. Several caspase activities, including those of caspases 1, 2, 3, 6, 8 and 9, were measured at each time point using peptide-fluorescence substrates. With the exception of caspase 3, no significant change or increase in caspase activity, along cell-cycle progression, was demonstrated in this study (Figure 2A). The activity of caspase 3 was increased about two-fold when cells were resided at G2/M transition. Most cells resided at the time interval right before the highest mitotic period (t = 10 and 11 h; Figure 2C bottom), and were ready to enter mitosis.

To further delineate and elaborate the time course of caspase 3 activation during the HeLa cell cycle, we have prepared different but more detailed time-course samples. Furthermore, cell cycle progression was examined by the mitotic index as described in the materials and methods section. Western blot analysis of the proteolytic cleavage products, which were representative of the active caspase forms, of caspase 1 and 3 was performed (Figure 2C). The results revealed that caspase 3, which was overexpressed 9 h after release from G1/S block, *i.e.* during the G2/M transition, underwent proteolytic cleavage, while caspase 1 did not (Figure 2C; t = 9 h) as cells transversed from the G2 phase to mitosis, as judged by the mitotic index. This was evident from the fact that, at nine hours, approximately 6% of the cells were mitotic, in contrast to the 25% that were mitotic at ten hours, suggesting that the G2-M transition of the HeLa cell cycle took place within the interval demarcated by these two time points. In addition, no detectable processing of caspase 8 and 9 was observed during the same cell cycle time course (data not shown). The up regulation of caspase 3 was not due to the loading artifact, as evidenced from caspase 1 Western analysis, with the finding consistent with the microarray data (Figure 1).[14] Taken together, we conclude that caspase 3 is activated at the G2/M transition and is independent of other caspase activities tested. Analysis of this data indicates the existence of a caspase 3 activation pathway, which does not involve the activation of either caspase 8 or 9, functioning as the initiator caspases.[6] It is, however, not clear whether other initiator caspases might regulate the activation of caspase 3 or whether caspase 3 is activated through a yet unidentified mechanism at the G2/M transition.

Figure 2. " (A) Time-course of caspase activity in samples of synchronized HeLa cells taken at different cell cycle time intervals as determined for caspases 1, 2, 3, 6, 8 and 9. Relative caspase activity is plotted in dependence upon time interval passed after release of cells from G1/S block. Description of synchronization and block release belongs to Material and Methods (release achieved by washing with drug-free medium) (B) Cell-cycle distribution as determined by flow cytometry. Relative proportions of cells in G1-, S- and G2/M phase are shown in dependence upon time interval after release from G1/S block. Sub-G1 represents the cells undergoing apoptosis where DNA is fragmentizedk. (C) Proteolytic cleavage of caspase 3 during G2/M transition as determined by Western blotting using anti-caspase 1 and 3 antibodies. T stands for the time (hours) after G1/S release. N represents control cells without thymidine treatment. The cell cycle progression was monitored by examination of the mitotic index. Active caspase 3, as illustrated by the presence of the processing form of caspase 3, was detected at the G2/M transition ($t = 9$ h). Note that there was no detectable active caspase 1 as judged by Western blotting.

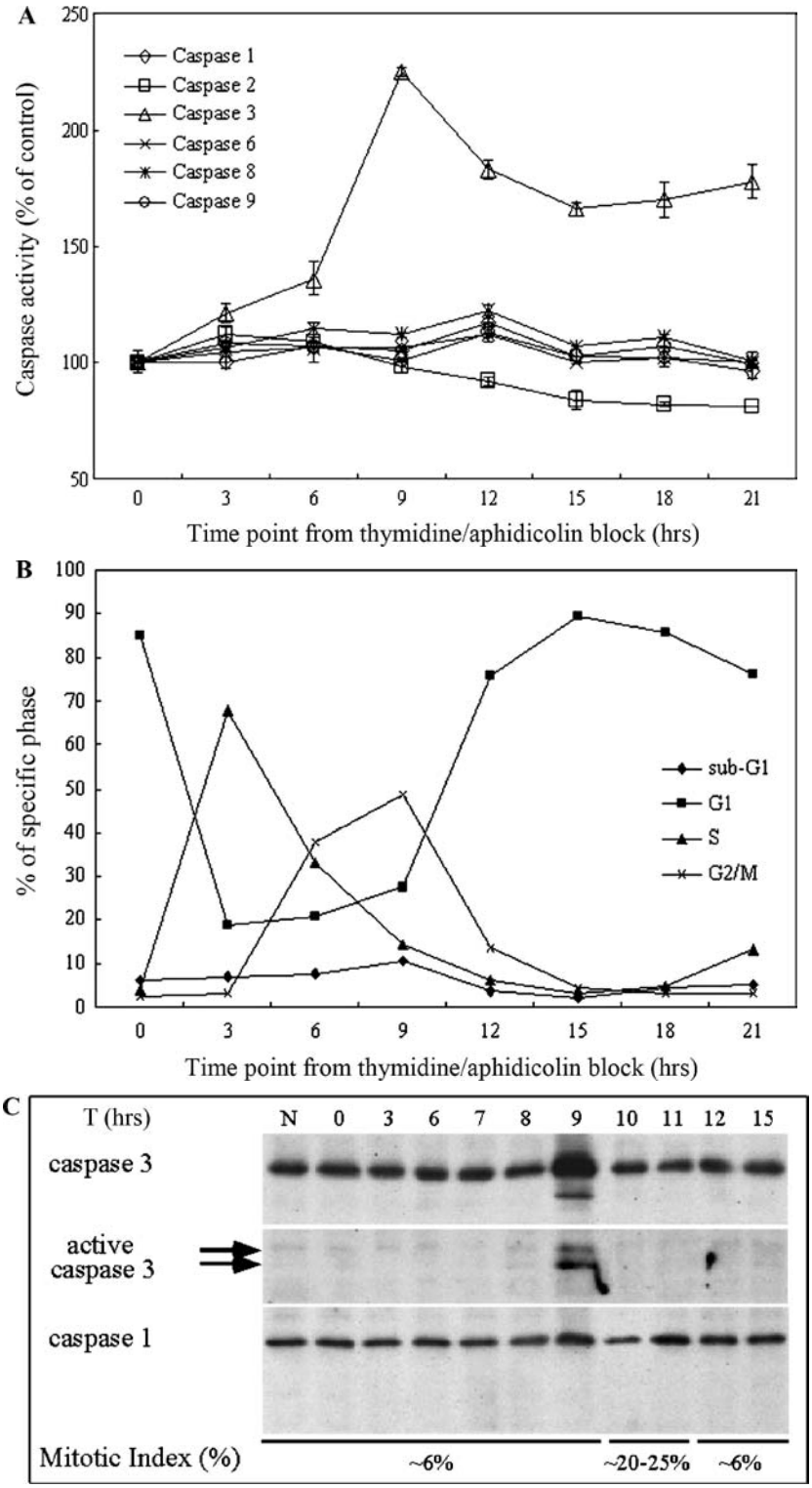
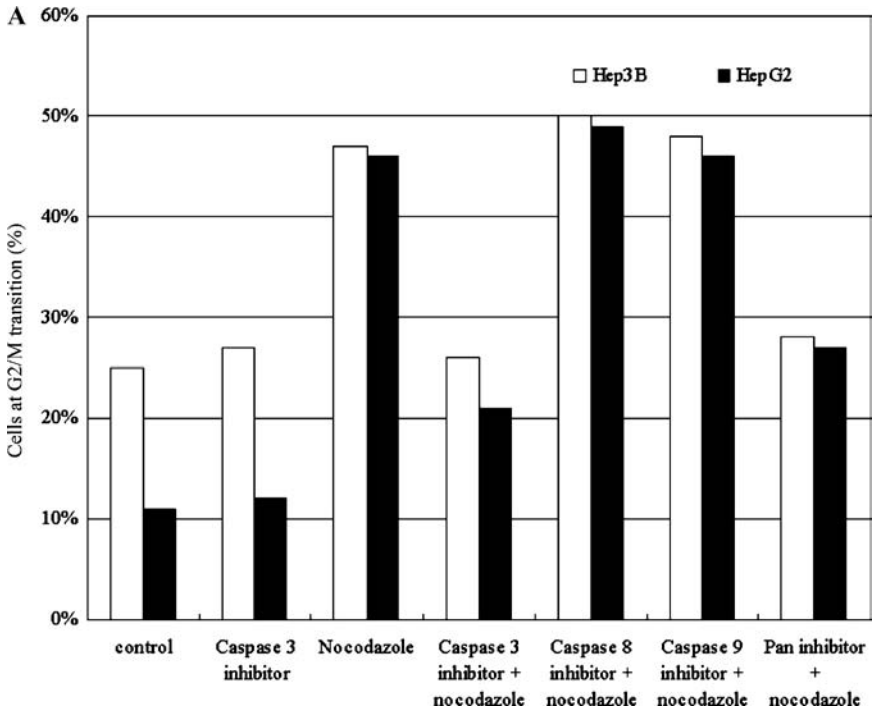
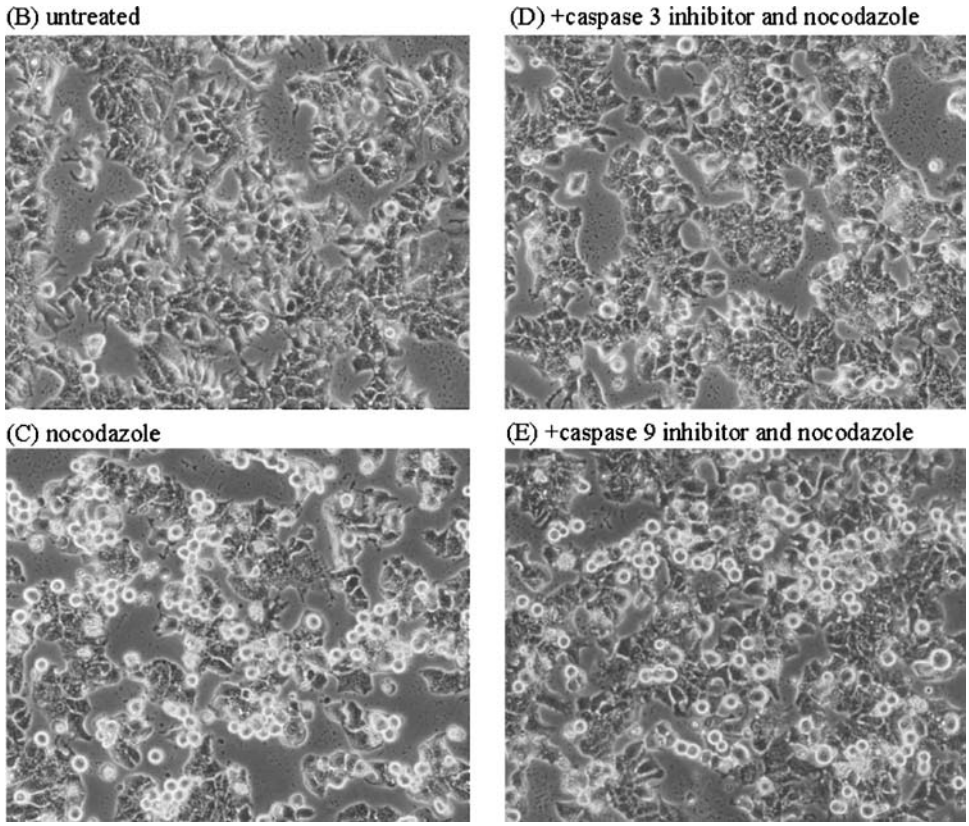


Figure 3. Inactivation of caspase 3 (or a caspase 3-like enzyme) abrogated the mitotic checkpoint in hepatoma cell lines. (A) Hep3B (white bar) or HepG2 (black bar) cells were pre-incubated with or without caspase 3, 8, 9, or pan inhibitors followed by with or without nocodazole treatment. Cells were collected and subjected to flow cytometry analysis, and only the relative number (%) of cells at G2/M is shown in order to simplify the pattern. The cell morphology of Hep3B (B), Hep3B incubated with nocodazole (C), and preincubation of Hep3B with caspase 3 (D) or 9 (E) inhibitor prior to nocodazole treatment was shown. The rounded morphology, evident in (C) and (E), was taken as an indication of cells in mitosis.





Caspase 3 (or a caspase 3-like enzyme) is involved in the mitotic checkpoint

It has been proposed that apoptosis coupling to surveillance mechanisms, such as checkpoint control, eliminates damaged and potentially harmful cells.[15] To further study the increased caspase 3 activity of cells at the G2/M transition in Hepatoma cell lines, we hypothesized that activation of caspase 3 might be involved in the regulation of mitotic-spindle checkpoint. If this were the case, interference with caspase 3 activity may perturb the functional mitotic checkpoint. In this study, nocodazole, which is an anti-microtubule drug that disrupts spindle assembly and activates a mitotic checkpoint,[16] was used to test this hypothesis. In line with the results of a previous study, nocodazole treatment of Hep3B cells caused mitotic arrest, as judged by FACS analysis (Figure 3A) and by changes in cell morphology, from flat (Figure 3B) to rounded shape (Figure 3C), typical for mitotic cells. The data imply that the mitotic checkpoint is intact when nocodazole-treated cells were blocked from completing mitosis due to spindle assembly disruption, *i.e.* accumulated at G2/M transition point. In contrast, pretreatment with caspase 3 or a broad-spectrum caspase inhibitor (pan caspase inhibitor) prior to nocodazole treatment did not arrest Hep3B cells at G2/M-transition point, as shown by FACS analysis (Figure 3A) and by examination of the morphological changes, with rounded cell morphology not exhibited (Figure 3D and data not shown, respectively). Importantly, this unanticipated role of caspase 3 was not due to drug treatment. This effect was absent when cells were pretreated with either caspase 3 inhibitor alone or inhibitors of caspase 8 and 9 combined with subsequent nocodazole treatment as illustrated by FACS analysis (Figure 3A) and by rounded-shape in cell morphology (Figure 3E). Most of morphologically rounded-shape cells were not apoptotic as judged by FACS analysis (less than 5% cells were apoptotic) (data not shown). The data strongly suggest the involvement of caspase 3 in mitotic checkpoint. Similar results were obtained when HepG2 cells cells were analyzed by the same assay (Figure 3A and data not shown).

Conclusion

In summary, we report that caspase 3 is upregulated and activated during the G2/M transition. In addition, the mitotic checkpoint, activated by nocodazole, is abrogated when caspase 3, but not initiator caspase 8 or 9, is inhibited. At present, it is not known how caspase 3 participates in the network of mitotic checkpoint. Nevertheless, the pre-activated caspase 3 may enable the surveillance system to response rapidly when cells are seriously damaged. Further analysis may advance our knowledge in the possible link between apoptosis and mitotic checkpoint.

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