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Award Number: DAMD17-99-1-9180

TITLE: Caspase deficiency: involvement in breast cancer
carcinogenesis and resistance

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REPORT DATE: July 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20021129 038

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 2002	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 01 - 30 Jun 02)	
4. TITLE AND SUBTITLE Caspase deficiency: involvement in breast cancer carcinogenesis and resistance		5. FUNDING NUMBERS DAMD17-99-1-9180	
6. AUTHOR(S) XiaoHe Yang, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) ENH Research Institute Evanston, Illinois 60201 Email: xiaohe-yang@ouhsc.edu		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) This project was proposed to study the correlation between caspase deficiency and breast cancer carcinogenesis and therapeutic resistance. During the third year, we continued to perform the functional screening of breast cancer cell lines treated with TNF- α and correlate their response with expression status of caspases in the cell lines detected in specific aim 1. Among the 7 breast cancer cell lines detected this year, BT-483, MDA-MB-134VI and MDA-MB-415 cells, which expressed lower levels of caspase 8, were resistant to TNF- α . When 19 breast cancer cell lines were treated with granzyme B, correlation between granzyme B induced apoptosis and caspase levels was less obvious as compared to TNF- α treatment. We have also analyzed sensitivity of 17 breast cancer cell lines treated with radiation and calculated the corresponding ID50 of each cell line. The Radio-sensitivities of these breast cancer cell lines were correlated to these cell lines to certain degree. As predicted, further specific analysis was suggested following these screening experiments. In addition, we found that caspase 3 had feedback actions on apical caspases and mitochondria in response to doxorubicin and TNF- α , implying the critical role of caspase 3 in therapeutic response of breast cancer cells.			
14. SUBJECT TERMS breast cancer, caspase, deficiency, resistance, therapy			15. NUMBER OF PAGES 31
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

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Introduction

Apoptosis (programmed cell death) is a fundamental process involved in homeostasis and the biochemical responses to different anti-tumor therapies. Aberrant expression of other apoptotic regulators, such as Bax- α and p53, has been associated with carcinogenesis and therapeutic resistance of breast cancer. Based on the fact that caspases are a group of proteases that mediate apoptotic execution, and that caspase 3 is deficient in MCF-7 breast cancer cells, this project was proposed to study the significance of caspase deficiency in breast cancer carcinogenesis and resistance. The specific aims are:

1. To determine the incidence and pattern of caspase deficiencies in breast cancer via screening for specific caspase expression in breast cancer derived cell lines, explant cultures and snap frozen human breast cancer tissue.
2. Correlation of caspase data (obtained in aim 1) with apoptosis induction using immune modulators (TNF- α , anti-Fas, GrB/Ad), chemotherapeutic agents and radiation on breast cancer derived cell lines and explant cultures.
3. Define the biologic role of specific caspase deficiencies via reconstitution of deficient caspases and comparative studies to define apoptosis induction in response to immune modulators, chemo- and radiation therapy.

In the past year, the PI moved from Evanston Northwestern Healthcare Research Institute/Northwestern University to University of Oklahoma Health Science Center to take a tenure track position as an Assistant Professor. Although the grant money is still in the process of transfer, we tried to continue the work and keep the influence of the transfer on the project progress to a minimum.

In the third year of this project, we performed the experiments as planned in the original proposal. The major task was to analyze the cell death of different breast cancer cell lines treated with chemotherapeutic agents and radiation (months 23-39). We also continued the functional screening of breast cancer cell lines in response to tumor necrosis factor- α (TNF- α) and granzyme/adenovirus (GrB/Ad) treatment. As it was planned, in depth analysis of the effect of caspase 3 deficiency on apoptosis in MCF-7 breast cancer cells was

also performed, which belongs to task 4 (months 30-48). Our results have been presented in a national meeting and being prepared for more publications.

Body

The progress of the project is reflected in the following aspects.

A. Functional screening of breast cancer cell lines in response to TNF- α and GrB/Ad treatment (specific aim 2, task 2)

According to the proposal, these experiments were designed to examine the susceptibility of different breast cancer cell lines in response to cytokines and granzyme B. The apoptotic response of the treated cells would be correlated with caspase expression profiles derived in specific aim 1.

1. Apoptosis of 7 breast cancer cell lines treated with TNF- α and CHX

In the last report, we reported the functional screening of 11 breast cancer cell lines treated with TNF- α and cycloheximide (CHX) (see the second year report). This year, we have continued the experiments. Breast cancer cell lines BT-483, MDA-MB-134VI, MDA-MB-415, MDA-MB-361, SK-BR-3, BT-20 and ZR-75-30 were treated with 80 ng/ml TNF- α plus 5 μ g/ml CHX for four hours. Apoptosis of the cells was determined using DAPI stain. Among these cell lines, BT-20 and Sk-BR-3 were very sensitive to TNF- α /CHX treatment (Fig. 1, Appendix A). Relatively, BT-483, MDA-MB-134VI and MDA-MB-415 were resistant to TNF- α /CHX treatment. ZR-75-30, which is a slow-growing cell line and sensitive to detachment, showed more apoptosis in both control and treated cells.

It has been reported that TNF- α induces apoptosis via death receptor activated caspase 8 pathway (1). It is interesting to notice that BT-20 and SK-BR-3 cells have higher levels of caspase 8 (Fig. 1 of appendix B in the first report and Fig. 2 of Appendix A in the second year report). BT-483, MDA-MB-134VI and MDA-MB-415 cells had lower levels of

caspase 8. In addition, the levels of caspase 3, 6 and 10 were also lower in these cells. These results suggest that caspase levels in breast cancer cells are correlated with TNF- α induced apoptosis.

2. Apoptotic responses of breast cancer cell lines treated with granzyme B/adenovirus (GrB/Ad)

Granzyme B (GrB) is a major component of cytotoxic granules released by natural killer (NK) cells and cytotoxic T lymphocytes. Our previous work indicated that caspase 3 deficiency was related to the resistance to GrB mediated apoptosis (2). To correlate caspase expression status and GrB mediated apoptosis, we treated different breast cancer cell lines with GrB and Ad (replication defective type 2 adenovirus was used to deliver GrB).

In addition to MCF-7 cells, which have been studied extensively in our previous work, we examined the apoptotic responses of 19 breast cancer cell lines treated with GrB/Ad. As shown in Fig. 2 Appendix A, MDA-MB-231, HI-578T, MDA-MB-436, MDA-MB-435S and MDA-MB-157 were very sensitive to GrB/Ad treatment. BT-474, ZR-75-1, MDA-MB-361, SK-BR-3 and MDA-MB-415 were relatively resistant to GrB/Ad treatment. The overall correlation between caspase expression status and GrB/Ad treatment was not as close as TNF- α treatment. This might be because that, as a protease by itself, GrB could trigger apoptosis at multiple points as compared to TNF- α . The efficiency of GrB uptake may also contribute to the apoptotic efficiency. This could be further studied in future research.

B. Analysis of the sensitivity of breast cancer cells to chemotherapeutic agent and radiation

Chemotherapy and radiotherapy are commonly used in breast cancer treatment. Apoptosis is involved in chemo- and radio-therapy mediated killing mechanisms. Aberrant apoptosis has been associated with therapeutic resistance. We found that MCF-7 cells, which are deficient in caspase 3, were resistant to these therapies. Reconstitution of caspase 3 in these cells sensitized them to the therapies ((3) and previous reports). We proposed to study the

correlation between caspase deficiency or expression status of caspases in breast cancer cells and their response to doxorubicin and radiation treatment.

This was planned as task 3 (months 23-29). We were ahead of the schedule in the previous year. We had examined the response of different breast cancer cell lines to chemotherapeutic agent doxorubicin (DOX) in the last annual report. We continued the work by assessing the response of breast cancer cell lines treated with radiation using MTT assay. Although it was designed to select limited numbers of cell lines with aberrant caspases, given the diverse caspase expression profiles and functional outcomes seen in TNF- α or GrB treated cells in different cell lines, we performed the examination on more cell lines to gain a comprehensive understanding of the correlation between caspase expression status and therapeutic responses.

To assess the cell death in response to radiation, breast cancer cell lines were irradiated at different doses (from 0.5 to 32 Gy) followed by viability determination using MTT assay. As shown in Fig. 3-6 Appendix A, the responses of different breast cancer cell lines treated with radiation varied significantly. MDA-MB-330, MDA-MB-134VI, MDA-MB-361 and ZR-75-30 cell lines were resistant to radiation. The survival fractions of these lines were still over 75% in the groups treated with 32 Gy of radiation, as compared to the other lines which had significant sensitivities at doses less than 10 Gy. Since all these lines are slow growing cell lines, other than apoptotic regulation, cell proliferation kinetics may have contributed to the response. Further study should be followed for these special cell lines.

ID50 or IC50 (dose or concentration of 50% inhibition) is usually used in the analysis of dose-effect response to cytotoxic treatment. However, it was not applicable to above-mentioned resistant lines because the survival fractions were above 50% at all the dosages used. For most of the remaining cell line, we performed model fitting and calculated the ID50 of these lines for sensitivity comparison. As summarized in Fig. 7 Appendix A, MDA-MB-231, MDA-MB-157, and MDA-MB-468, which had lower ID50, were very sensitive to radiation. In contrast, MDA-MB-453, MDA-MB-415 and BT-474 were relatively resistant.

In context with caspase expression status, MDA-MB-231, MDA-MB-157, and MDA-MB-468 cells, which were sensitive to radiation, expressed high levels of caspases. MDA-MB-134VI and MDA-MB-415, which were resistant to radiation, expressed low levels of caspases 2, 3, 6 and 8. Interestingly, caspase levels in BT-474 and MDA-MB-453 cells, which are also relatively resistant to radiation, were high, as compared to MDA-MB-134VI and MDA-MB-415 cells.

The results suggested that low levels of caspase expression, like that found in MDA-MB-134VI and MDA-MB-415 cells, may contribute to radiation resistance. Obviously, caspase expression was not the sole factor contributing to the resistance. As suggested by these screening data, the contribution of specific caspase to radiation resistance should be addressed by specific blocking or overexpression followed by functional assay.

C. Caspase 3 deficiency in breast cancer ----- feedback action of caspase 3 on apical factors

The specific aim 3 of this project was to define the biological role of specific caspase deficiencies via specific reconstitution of corresponding caspase. These was planed in ask 4 (months 30-48).

Previously, we reconstituted caspase 3 in MCF-7 breast cancer cells, which are deficient in caspase 3. With the caspase 3 specific cell line model, we have demonstrated that caspase 3 is critical in granzyme B, chemotherapy and radiotherapy induced apoptosis. When we studied the role of caspase 3 in radiation induced apoptosis, our results suggested that caspase 3 might have feedback action on apical factors. Therefore, we performed the following experiments to investigate the feedback action of caspase 3 on doxorubicin and TNF- α induced apoptosis.

1. Apical caspases 2, 8, 9 and 10 could be processed by caspase 3 in a pure biochemical system

The major apical factors that are up-stream of caspase 3 in apoptosis cascade are the apical caspases and the factors released from mitochondria (4). We first looked at the feed back action on apical caspases, including caspases 2, 8, 9 and 10 by detecting the activation of these caspases in MCF-7/pV control cells and MCF-7/C3 (caspase 3 reconstituted) cells. However, even if we could see the difference of apical caspase activation/processing between the two cell lines, the differences could be the result of many direct or indirect reactions. To test whether caspase 3 could directly activate/process these apical caspases, we prepared caspases 2, 8, 9 and 10 by in vitro transcription and translation. These apical caspases were treated with purified caspase 3 individually. As shown in Fig. 8 Appendix A, our result indicated that, in pure biochemical system, apical caspases 2, 8, 9 and 10 could be the substrates of caspase 3, as indicated by the cleavage products. Among them, caspase 9 was a preferred substrate of caspase 3, followed by caspases 2, 8, 9 and 10.

2. Processing/activation of apical caspases by cytosolic extracts from MCF-7/pv and MCF-7c3 cells.

We then compared the activation of the apical caspase by cytosolic extracts between MCF-7/pv and MCF-7/c3 cells treated with doxorubicin or TNF- α . In vitro transcription and translated apical caspases were treated with the cell lysate made from stimulated cells. Responses of caspases 2, 8, 9 and 10 to apoptotic cytosolic extract from MCF-7/c3 cells, which were MCF-7 cells reconstituted with caspase 3, were similar to treatment with purified caspase 3 (Fig. 9-12, Appendix A). The apical caspases were not activated by the lysate made from MCF-7/pv cells (caspase 3 deficient). Caspase 3 dependent activation of these caspases in the in vitro system was further demonstrated by blockage with DEVD-CHO, a caspase 3 specific peptide inhibitor, but not with YVAD-CHO, an inhibitor of caspase 1 like caspases. These results indicated that in doxorubicin or TNF- α treated breast cancer cells, activation of apical caspases would be enhanced in the presence of caspase 3. Use of the peptide inhibitors demonstrated that the activation of the apical caspases was caspase 3 specific.

3. Activation of apical caspases by caspase 3 in whole cells system

The *in vitro* system demonstrated the biochemical feasibility and specificity of apical caspase activation by caspase 3. However, cellular structure and compartmentalization may also affect the possible action of caspase 3 on apical caspases. We then detected the activation of caspases 2, 8 and 9 in the lysate made directly from doxorubicin or TNF- α treated cells (Fig. 13-14, Appendix A). In whole cell system, processing/activation of caspases 8 and 9 was enhanced in doxorubicin or TNF- α treated MCF-7/c3 (caspase 3 reconstituted) cells, as compared to caspase 3 deficient MCF-7/pv cells. Caspase 2 activation was minimal in both cell lines under the given conditions. Discrepancy of caspases 2 and 8 between cell free and whole cell systems suggested that the structural compartment might contribute to caspase 3 mediated feedback action.

4. Feedback action of caspase 3 on cytochrome c release from mitochondria

Cytochrome c release from mitochondria is one of the major steps in the activation of effector caspases, such as caspase 3. In our previous study, we found that caspase 3 enhance depolarization of mitochondria (see last year report). Therefore, we hypothesized that caspase 3 would have feed back action on cytochrome c release. We compared cytochrome c released to the cytosol from doxorubicin treated cells and found that cytochrome c release was indeed increased in treated MCF-7/c3 cells (Fig. 15, Appendix A), suggesting that caspase 3 also had feedback action on mitochondria mediated apoptotic activities.

Taken together, our results indicated that caspase 3 has feedback action on apical factors, including apical caspases and mitochondrial activities. The work extended our understanding of the role of caspase 3 in apoptosis. It suggested that deficiency of caspase 3 would result in worse consequence on cellular response to anti-tumor mechanisms or therapies.

Key research Accomplishments

- We have assessed the apoptotic response of 7 breast cancer cells lines treated with TNF- α and CHX (Continuation of last year's work).

- We have assessed the apoptotic response of 19 breast cancer cell lines treated with GrB.
- We have analyzed the sensitivity of 17 breast cancer cell lines to radiation (to doxorubicin was done last year).
- We have analyzed enzymatic activity of caspase 3 on apical caspases 2, 8, 9 and 10 in a biochemical system.
- We have analyzed the cleavage/activation of in vitro transcribed/translated apical caspases 2, 8, 9 and 10 by cell lysate from MCF-7/pv and MCF-7/c3 cells treated with doxorubicin or TNF- α .
- We detected the activation of apical caspases 2, 8 and 9 in MCF-7/pv and MCF-7/c3 cells treated with doxorubicin or TNF- α .
- We compared cytochrome c release from mitochondria of MCF-7/pv and MCF-7/c3 cells treated with doxorubicin or TNF- α .

Reportable Outcomes

Meeting Abstract:

Yang XH, Liu XS, Yang SH, Khandekar JD, Froelich CJ, and Thor AD, Feedback action of caspase 3 on apical factors, Molecular targets and cancer therapeutics, AACR-NCI-EORTC International Conference, Miami, Florida, Oct. 2001

Conclusion

Summary

During the third year of the project, we have continued our progress toward defining the role of caspase deficiencies in breast cancer carcinogenesis and resistance to therapeutics. First we continued to assess the apoptotic response of breast cancer cell lines treated with TNF- α . Correlating response with caspase expression status of the breast cancer cell lines has suggested that caspase 8 status is relative related to TNF- α induced response, which is

consistent with the notion that caspase 8 is the major apical caspase in TNF- α induced apoptosis.

Secondly, we evaluated different breast cancer cell lines treated with granzyme B. We found that granzyme B induced apoptosis varied from one cell line to another. The correlation between caspase expression status and apoptotic responses was not as close as TNF- α treatment. This might be due to the fact that granzyme B itself is an enzyme which can activate apoptosis at multiple points of caspase cascade, and that the efficiency of granzyme B uptake by different cells may also contribute to the process.

Thirdly, evaluation of the sensitivity of different breast cancer cell lines treated with radiation suggested that caspases expression status in some of the cell lines, such as MDA-MB-134VI and MDA-MB-415 cells, might be correlated with radiation resistance. However, the specific contribution of individual caspase has to be addressed in a specific model.

Along this line, we continued our study using caspase 3 deficient/reconstituted MCF-7 cell line models. As demonstrated by both in vitro and in vivo results, we found that caspase 3 had feedback action on apical caspases and mitochondria. The results suggested that caspase 3 function is more important in therapeutically induced apoptosis and implies that defects in caspase 3 have a broader negative effect on breast carcinogenesis and therapeutic resistance.

“So what “ section

To date, we have examined the expression of 10 caspases in a collection of breast cancer cell lines and caspase 3 expression in some human breast cancer tissues. We have performed functional screening of those breast cancer cell lines with TNF- α , granzyme B, radiation and the chemotherapeutic agent doxorubicin. Our results have shown that caspase 3 was deficient in MCF-7 breast cancer cells. Although we did have not detected completely deletion of a caspase in other cell lines, like the caspase 3 deficiency in MCF-7

cells, we have detected decreased caspase expression in other breast cancer cell lines (see the first year annual report). Correlation between caspase 3 levels and resistance to apoptosis induced by different stimuli was observed. Our results are valuable references for further characterization of caspase expression and apoptotic resistance. With the rapid growth in the studies of apoptosis, however, we now know that caspases are a large family (14 caspases have been identified) and functional overlapping is involved in apoptosis. Some caspases are more important than the others in apoptosis execution. Indeed, our result suggested that the protein levels of caspases 3, 6 and 8 are closely related to apoptotic responses. Therefore, detailed correlation between individual caspase and the resistance should be addressed specifically. Based on our screening results, we will select the cell lines resistant to certain stimuli, like TNF- α or doxorubicin, to investigate the biochemical process between low caspase expression and the resistant phenotype we observed. Detection of the cleavage of caspase substrates, such as lamin B and DNA fragmentation factor will reveal more information related to this issue. Another aspect to follow is detection of caspase expression in human breast cancer tissues. Although deletion of extremely low levels of caspase expression was not significant in cell lines, our preliminary data from clinical samples found that 17% of breast cancers express low or non-detectable caspase 3. We will extend our detection to gain more information on caspase status in breast cancer samples. Other than deletion of a whole caspase molecule, point mutation should also be considered for further investigation on this issue (5).

By reconstitution of caspase 3 in MCF-7 cells, we have demonstrated that caspase 3 plays a critical role in apoptosis induced by different therapies. It strongly suggested that caspase 3 deficiency would contribute significantly to therapeutic resistance, which is consistent with our hypothesis. Using our cell line model, our results also demonstrated some basic mechanisms in apoptotic pathway, such as the feedback action of caspase 3. Our work is among the leading groups in this area. As planned in specific aim 3, more work on reconstituted cells will be done for in depth study of the role of caspases in breast carcinogenesis and therapeutic resistance.

Although the PI moved to the new institution during the third year of this project, our progress, relative to our original plan, is basically on schedule. At the same time, we also realize that some experiments are needed to complete the original plan or explain the observations we observed from our experiments. If it is necessary, we will apply for a no cost extension to fulfill the necessary experimental design and draw a comprehensive conclusion for this project. Clearly, with the results generated from this project, much more experiments could be followed.

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5. Schwartz, S., Jr., Yamamoto, H., Navarro, M., Maestro, M., Reventos, J., and Perucho, M. Frameshift mutations at mononucleotide repeats in caspase-5 and other target genes in endometrial and gastrointestinal cancer of the microsatellite mutator phenotype, *Cancer Res.* 59: 2995-3002, 1999.

APPENDICES:

Appendix A:

Results generated in the studies of:

- a. Functional screening of breast cancer cell lines in response to TNF- α and GrB/Ad
- b. Analysis of the sensitivity of breast cancer cell lines in response to radiation
- c. Feedback action of caspase 3 on apical caspase

Appendix B:

Abstract: Feedback action of caspase 3 on apical caspase

Presented at the Conference of Molecular targets and cancer therapeutics, AACR-NCI-EORTC International Conference, Miami, Florida, Oct. 2001

Appendix A

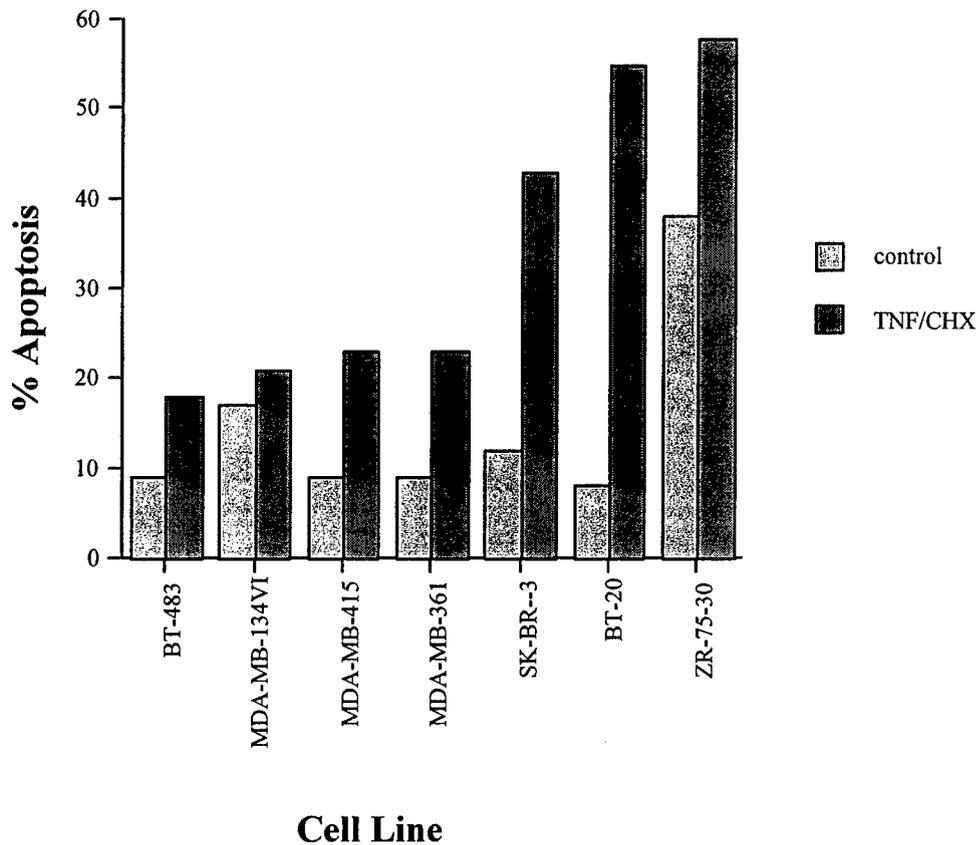


Fig. 1. Apoptosis in breast cancer cell lines treated with TNF- α

The cells of individual cell line were treated with medium alone (control) or 80 ng/ml TNF- α plus 5 μ g/ml cycloheximide for 4 hours. Apoptosis was analyzed using DAPI staining.

Appendix A

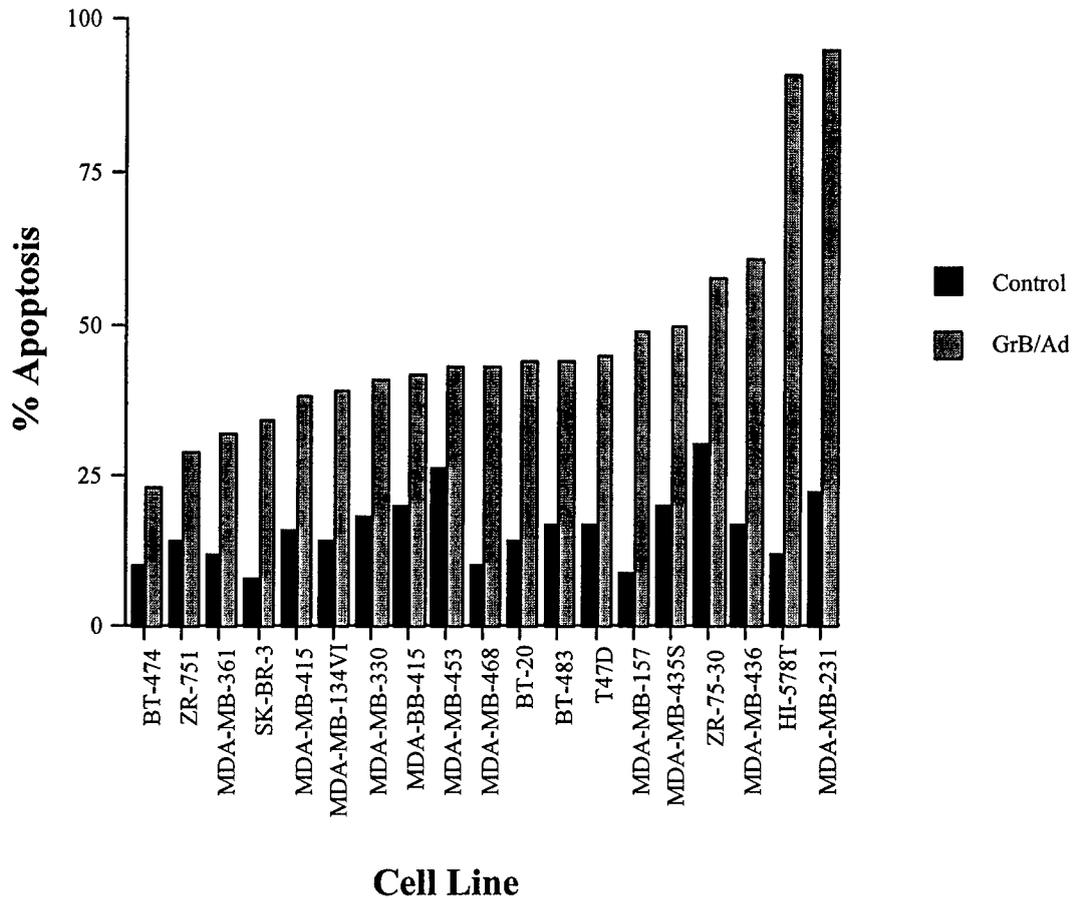


Fig. 2. Apoptosis in breast cancer cell lines treated with granzyme B

The cells of individual cell line were treated with medium alone (control) or 1 $\mu\text{g/ml}$ granzyme B (GrB) plus 50 PFU adenovirus (Ad) for 4 hours. Apoptosis was analyzed using DAPI staining.

Appendix A

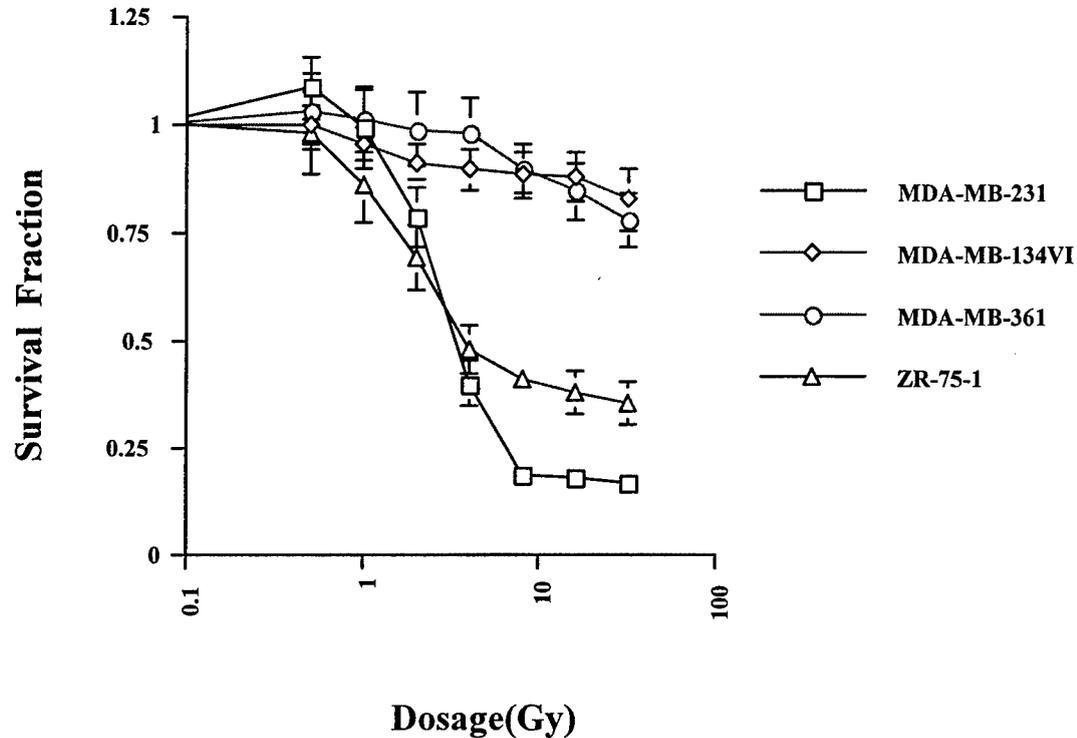


Fig. 3. Survival fractions of MDA-MB-231, MDA-MB-134VI, MDA-MB-361 and ZR-75-1 breast cancer cell lines in response to radiation

Different breast cancer cell lines were irradiated at the indicated dosage. Survival fractions were determined using MTT assay 6 days post radiation. Each point in the figure is the average survival fractions of three experiments. In each experiment, 8 parallel samples were tested for any concentration treatment.

Appendix A

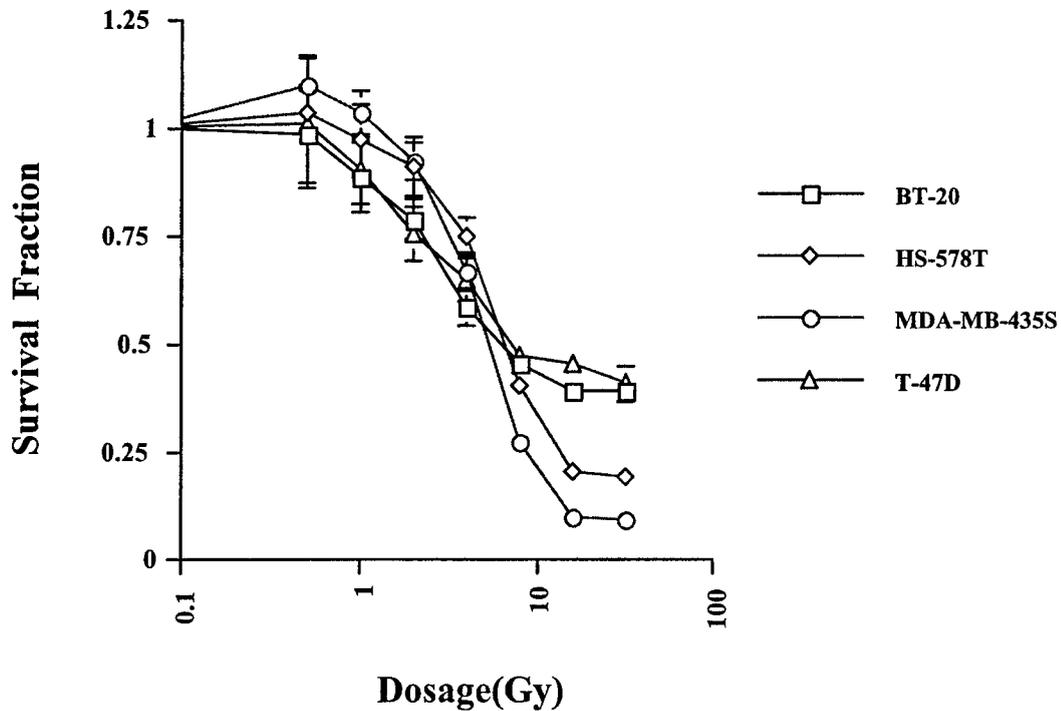


Fig. 4. Survival fractions of BT-20, HS-578T, MDA-MB-435s and T47D breast cancer cell lines in response to radiation

Different breast cancer cell lines were irradiated at the indicated dosage. Survival fractions were determined using MTT assay 6 days post radiation. Each point in the figure is the average survival fractions of three experiments. In each experiment, 8 parallel samples were tested for any concentration treatment.

Appendix A

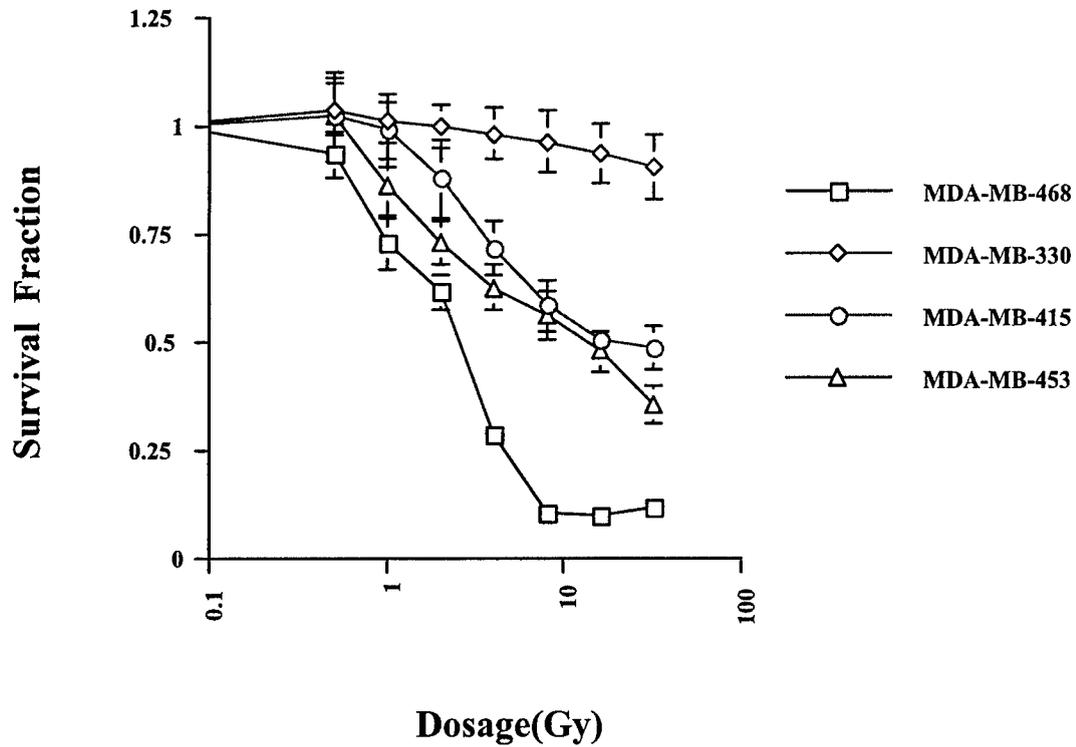


Fig. 5. Survival fractions of MDA-MB-468, MDA-MB-330, MDA-MB-415 and MDA-MB-453 breast cancer cell lines in response to radiation

Different breast cancer cell lines were irradiated at the indicated dosage. Survival fractions were determined using MTT assay 6 days post radiation. Each point in the figure is the average survival fractions of three experiments. In each experiment, 8 parallel samples were tested for any concentration treatment.

Appendix A

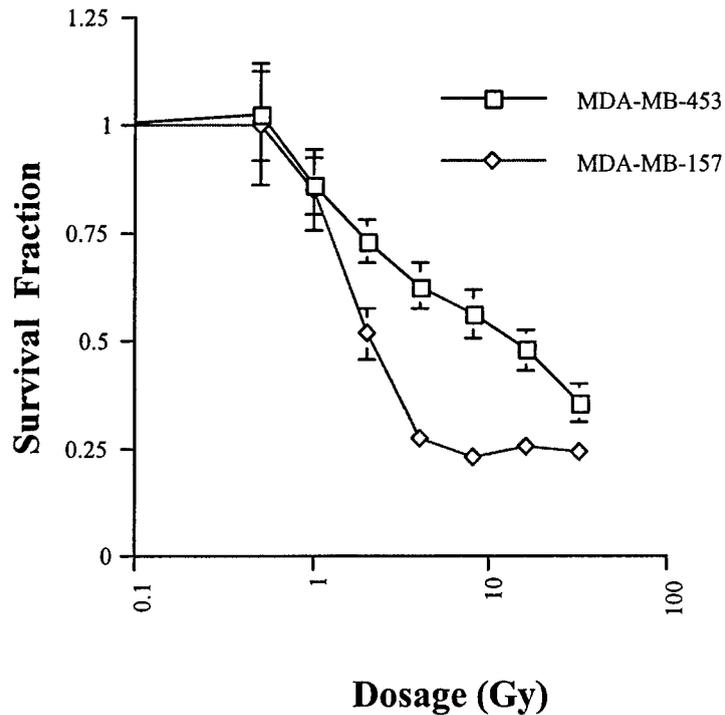


Fig. 6. Survival fractions of MDA-MB-157 and MDA-MB-453 breast cancer cell lines in response to radiation

Different breast cancer cell lines were irradiated at the indicated dosage. Survival fractions were determined using MTT assay 6 days post radiation. Each point in the figure is the average survival fractions of three experiments. In each experiment, 8 parallel samples were tested for any concentration treatment.

Appendix A

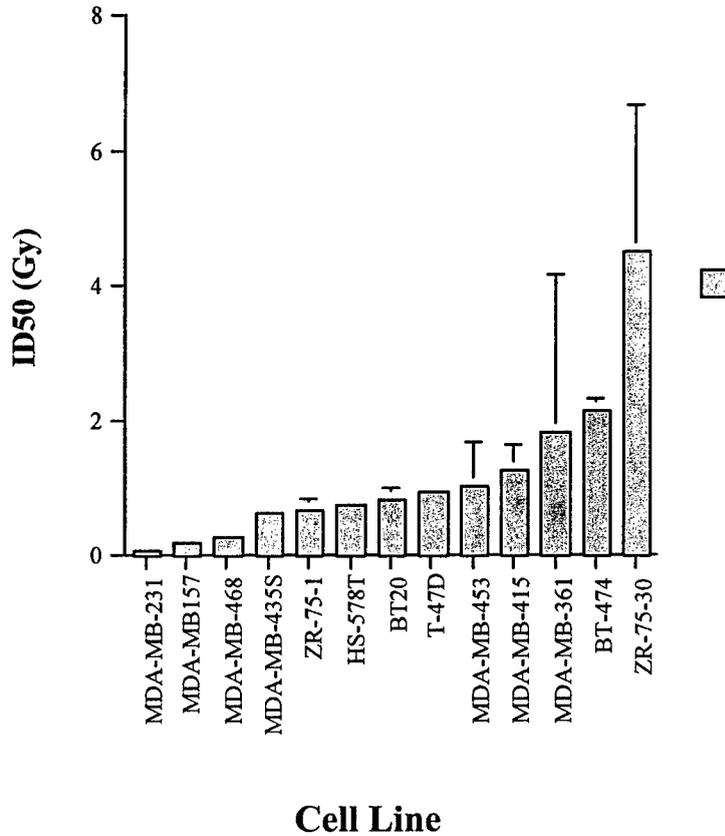


Fig. 7. ID50 of breast cancer cell lines in response to radiation

Breast cancer cell lines listed above were irradiated at doses ranging from 0.5 to 32 Gy. The survival fractions were detected 6 days post radiation using MTT assay. ID50 of each cell line was calculated based on the survival fractions of dose-effect response of the irradiated cells. The results for each cell line were repeated in three separate experiments.

Appendix A

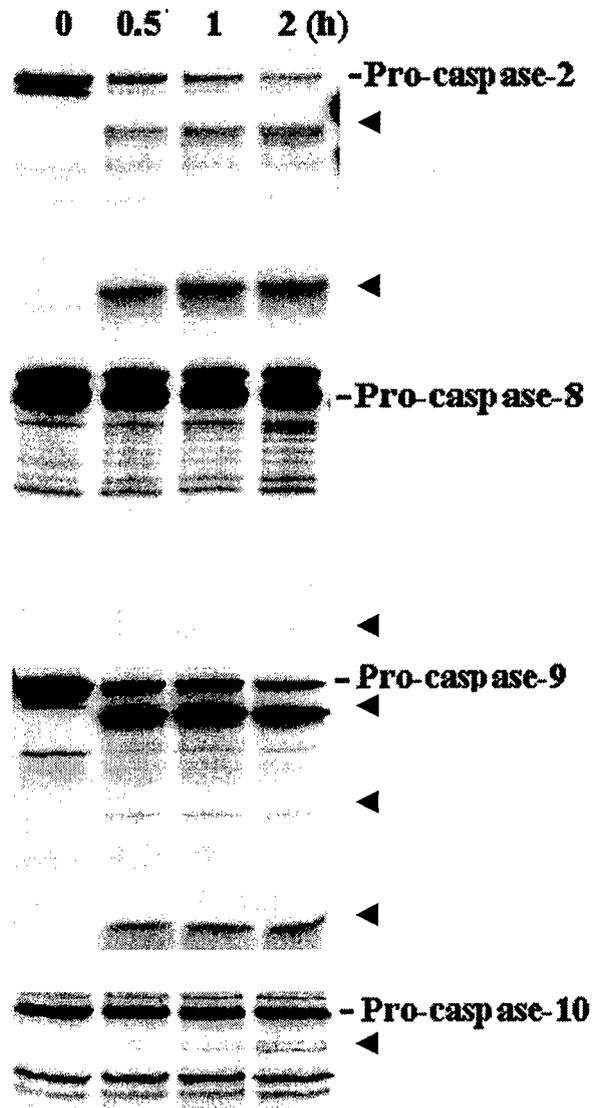


Fig. 8. Processing/activation of caspases 2, 8, 9, and 10 by purified caspase 3 in vitro.

³⁵S labeled caspases 2, 8, 9 and 10 were prepared using in vitro transcription and translation (TNT) system. Approximately 100 ng of TNT product was treated with 100 nM of purified caspase 3 for times indicated. The processing was analyzed by SDS-PAGE and autoradiography. Arrows indicate the cleaved products of pro-enzymes. Relatively, caspase 9 was the preferred substrate of caspase 3, followed by caspases 2, 8 and 10.

Appendix A

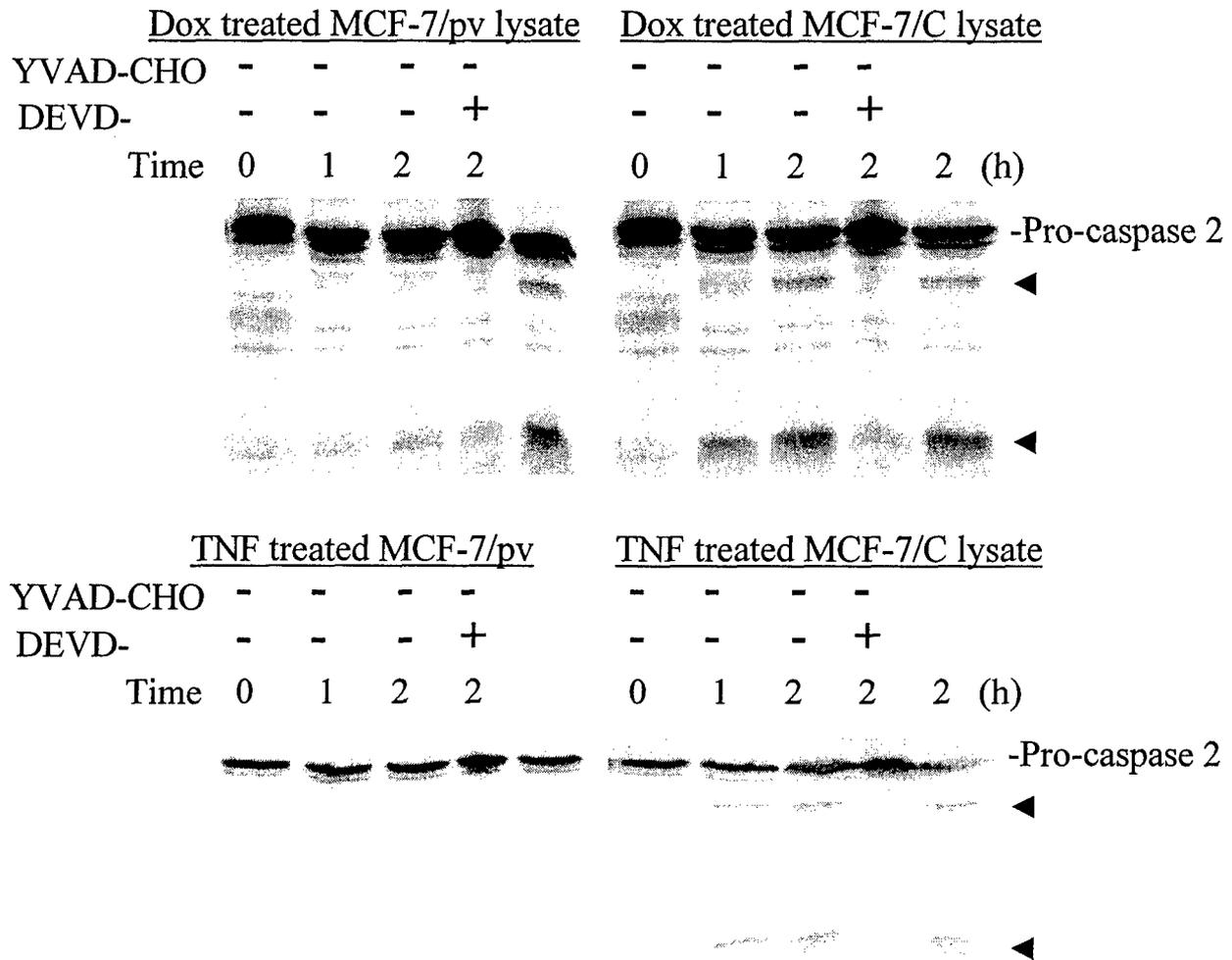


Fig. 9. Processing of caspases 2 by apoptotic cytosolic extract from caspase 3 deficient MCF-7/pv and caspase 3 reconstituted MCF-7/c cells.

Apoptotic cytosolic extract was prepared from MCF-7/pv and MCF-7/c cells treated with 5 μ M of doxorubicin for 20 hrs or 80 ng of tumor necrosis factor- α (TNF- α) and 5 μ g of cycloheximide (CHX) for 4 hrs, respectively. Approximately 100 ng of 35 S labeled caspase 2 was incubated with 30 μ g of cytosolic extract, in the presence or absence of 50 μ M of DEVD-CHO or YVAD-CHO, for times indicated. The processing was analyzed by SDS-PAGE and autoradiography. Arrows indicate the cleaved products of pro-enzymes.

Appendix A

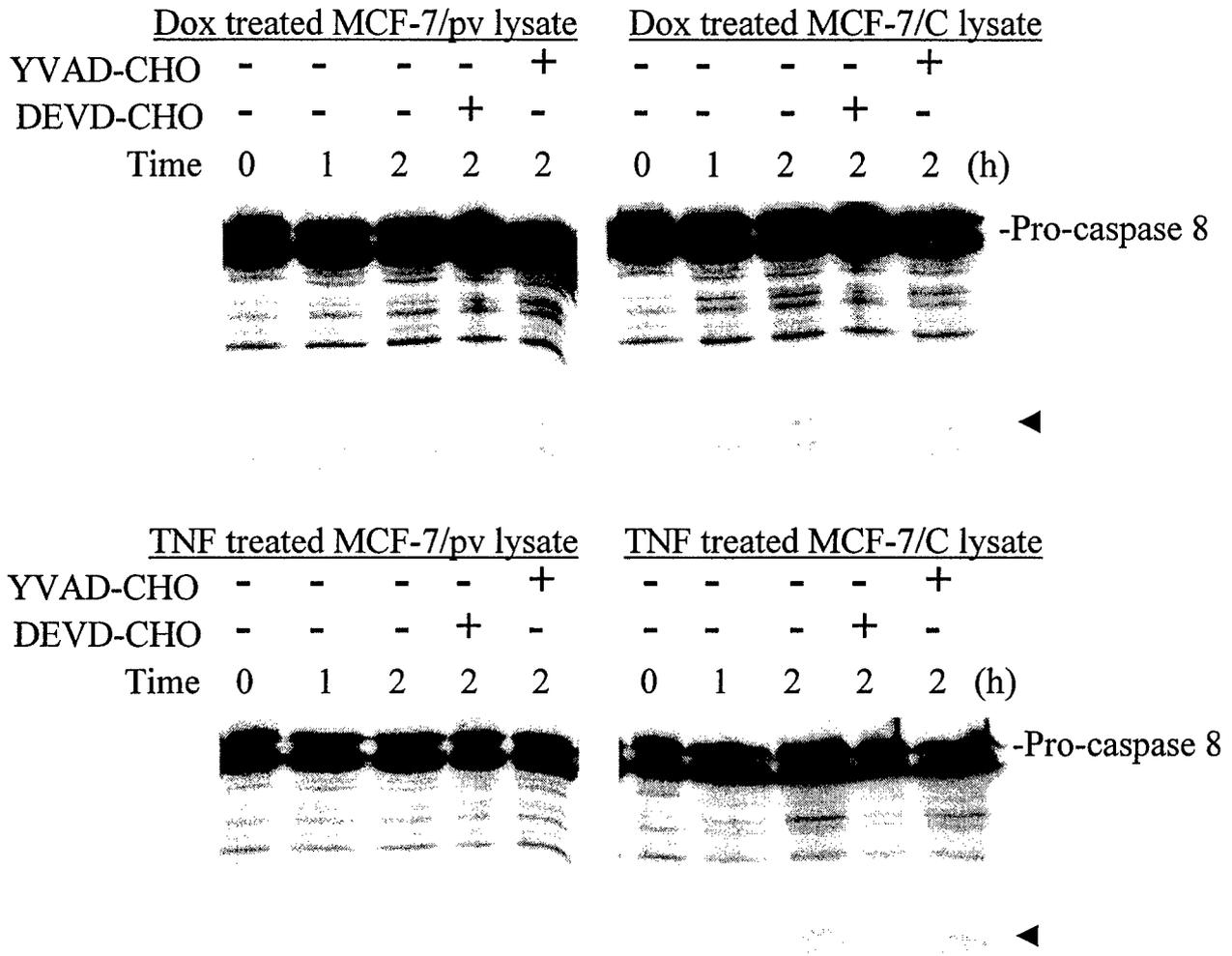


Fig. 10. Processing of caspases 8 by apoptotic cytosolic extract from caspase 3 deficient MCF-7/pv and caspase 3 reconstituted MCF-7/c cells.

Apoptotic cytosolic extract was prepared from MCF-7/pv and MCF-7/c cells treated with 5 μ M of doxorubicin for 20 hrs or 80 ng of tumor necrosis factor- α (TNF- α) and 5 μ g of cycloheximide (CHX) for 4 hrs, respectively. Approximately 100 ng of 35 S labeled caspase 8 was incubated with 30 μ g of cytosolic extract, in the presence or absence of 50 μ M of DEVD-CHO or YVAD-CHO, for times indicated. The processing was analyzed by SDS-PAGE and autoradiography. Arrows indicate the cleaved products of pro-enzymes.

Appendix A

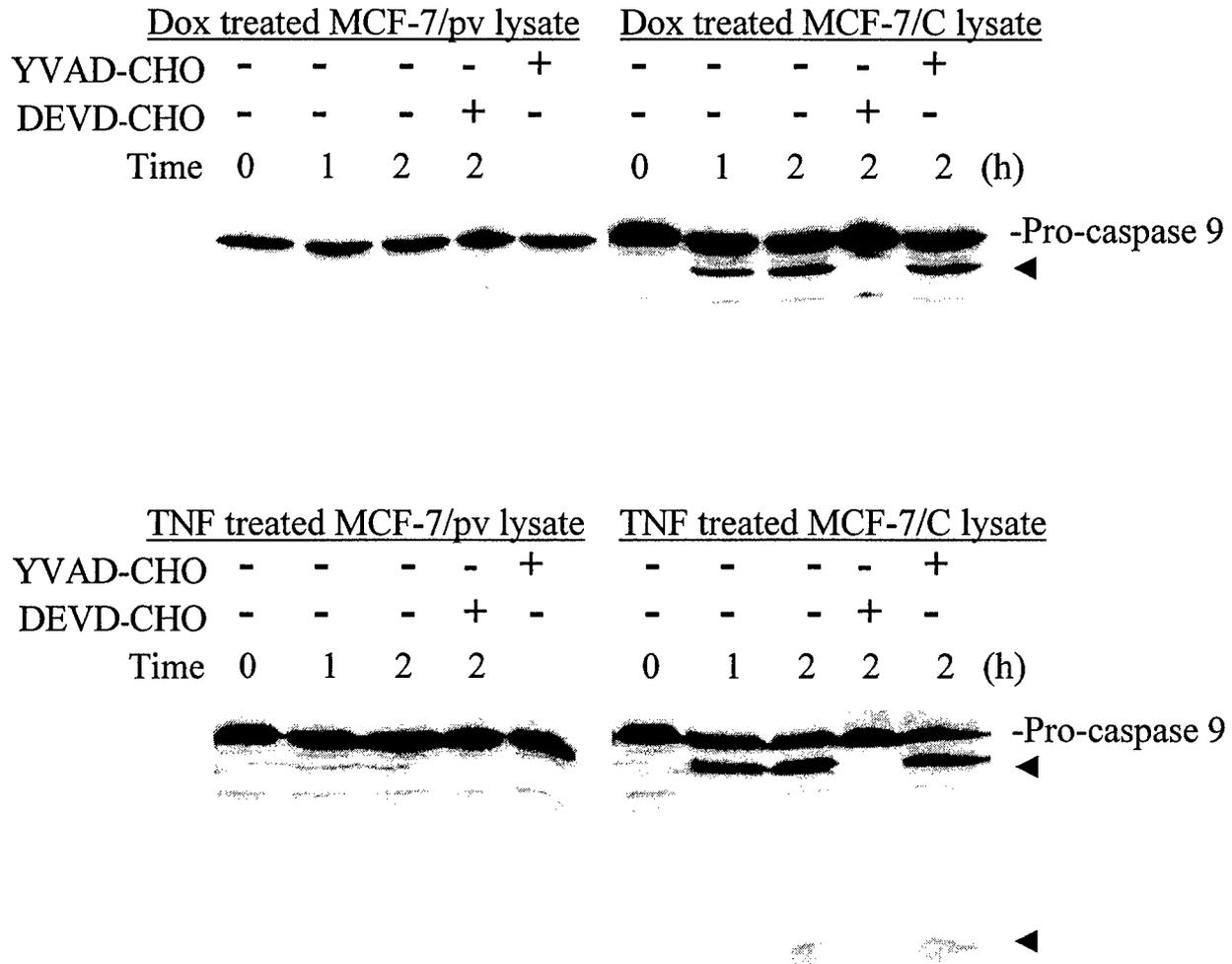


Fig. 11. Processing of caspases 9 by apoptotic cytosolic extract from caspase 3 deficient MCF-7/pv and caspase 3 reconstituted MCF-7/c cells.

Apoptotic cytosolic extract was prepared from MCF-7/pv and MCF-7/c cells treated with 5 μ M of doxorubicin for 20 hrs or 80 ng of tumor necrosis factor- α (TNF- α) and 5 μ g of cycloheximide (CHX) for 4 hrs, respectively. Approximately 100 ng of 35 S labeled caspase 9 was incubated with 30 μ g of cytosolic extract, in the presence or absence of 50 μ M of DEVD-CHO or YVAD-CHO, for times indicated. The processing was analyzed by SDS-PAGE and autoradiography. Arrows indicate the cleaved products of pro-enzymes.

Appendix A

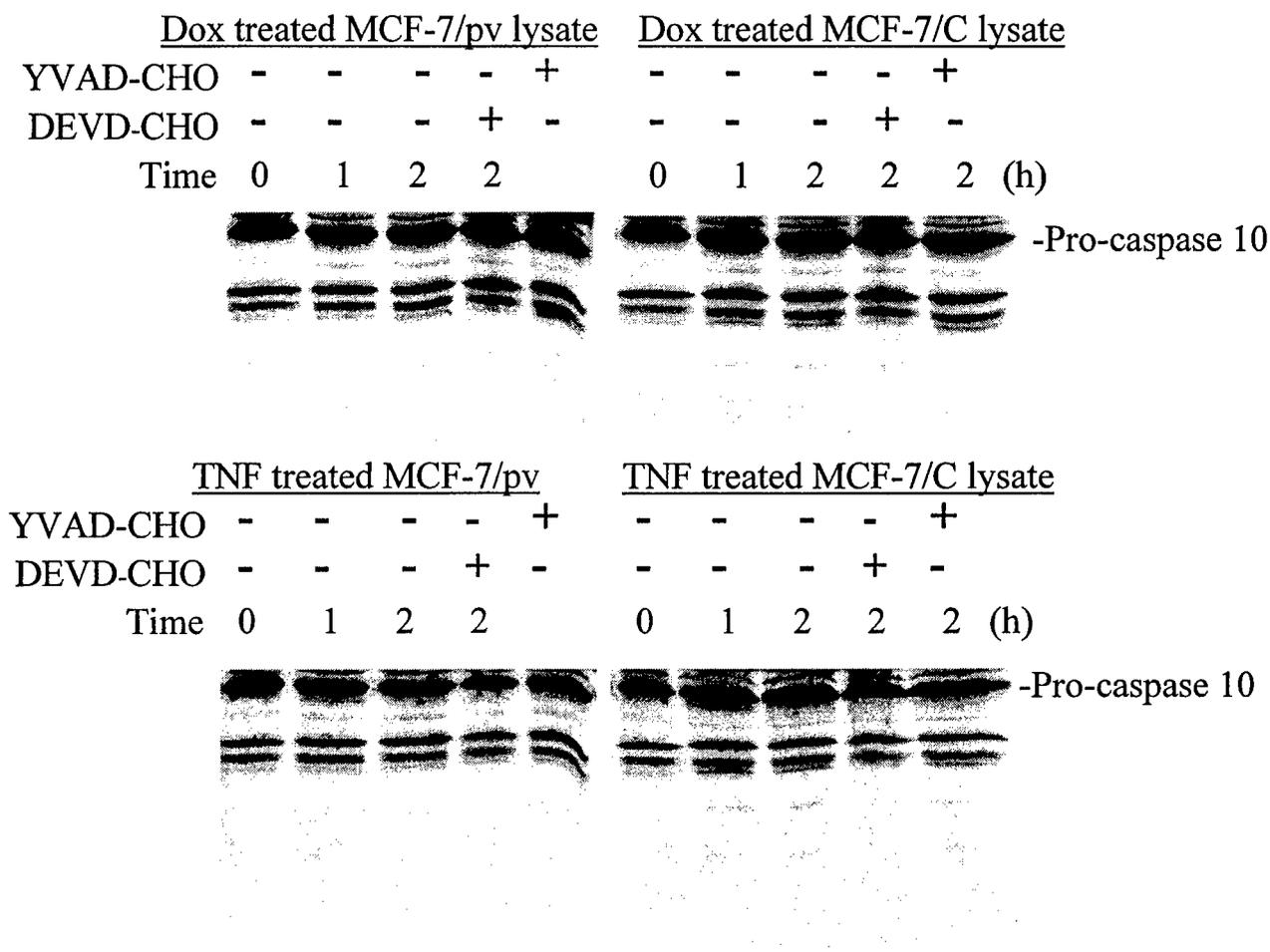


Fig. 12. Processing of caspases 10 by apoptotic cytosolic extract from caspase 3 deficient MCF-7/pv and caspase 3 reconstituted MCF-7/c cells.

Apoptotic cytosolic extract was prepared from MCF-7/pv and MCF-7/c cells treated with 5 μ M of doxorubicin for 20 hrs or 80 ng of tumor necrosis factor- α (TNF- α) and 5 μ g of cycloheximide (CHX) for 4 hrs, respectively. Approximately 100 ng of 35 S labeled caspase 10 was incubated with 30 μ g of cytosolic extract, in the presence or absence of 50 μ M of DEVD-CHO or YVAD-CHO, for times indicated. The processing was analyzed by SDS-PAGE and autoradiography. Arrows indicate the cleaved products of pro-enzymes.

Appendix A

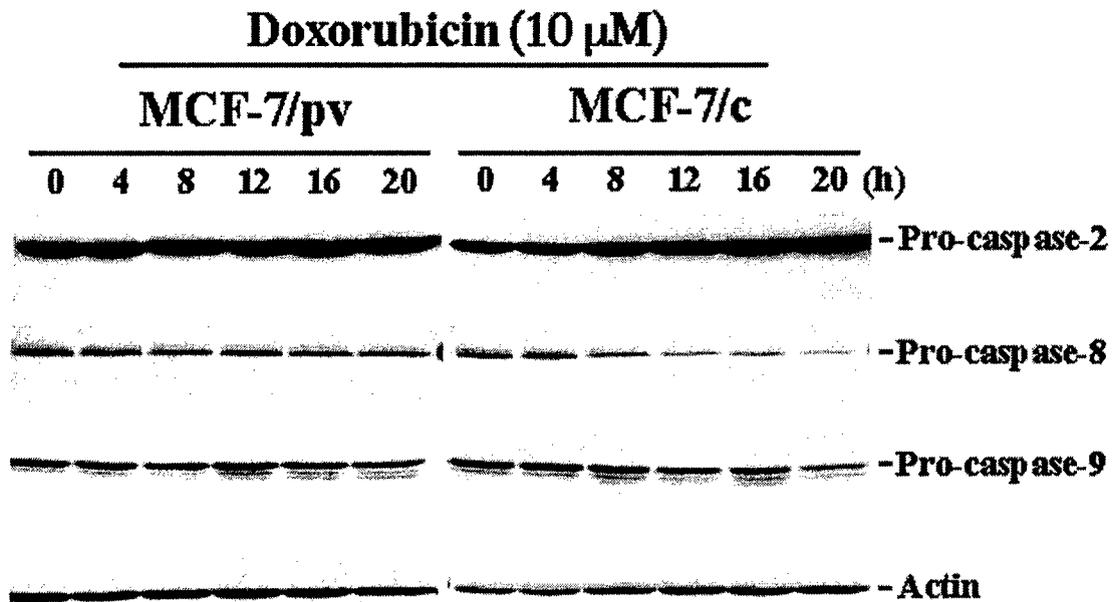


Fig. 13. Activation of caspases 8 and 9, but not caspase 2, was enhanced in doxorubicin treated MCF-7/c cells, as compared to MCF-7/pv cells.

MCF-7/pv (caspase 3 deficient) and MCF-7/c (caspase 3 reconstituted) cells were treated with 10 μ M doxorubicin at indicated concentrations for 4 hrs. Caspases 2, 8, 9 and actin were detected using Western blot

Appendix A

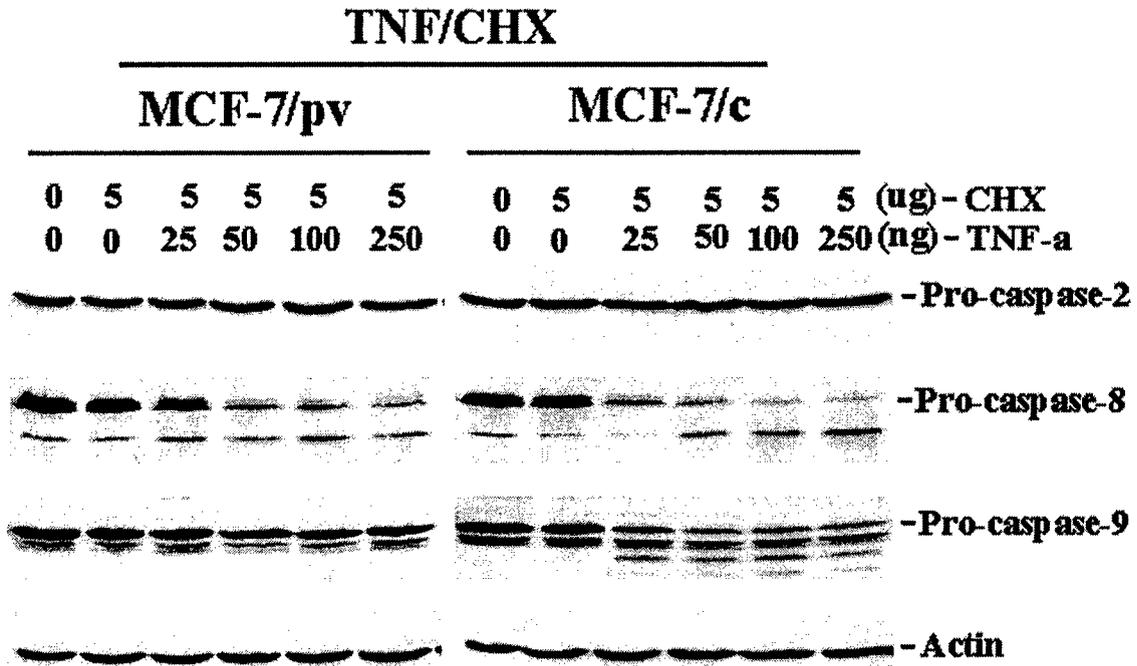


Fig. 14. Activation of caspases 8 and 9, but not caspase 2, was enhanced in TNF- α treated MCF-7/c cells, as compared to MCF-7/pv cells.

MCF-7/pv (caspase 3 deficient) and MCF-7/c (caspase 3 reconstituted) cells were treated with TNF- α and cycloheximide at indicated concentrations for 4 hrs. Caspases 2, 8, 9 and actin were detected using Western blot

Appendix A

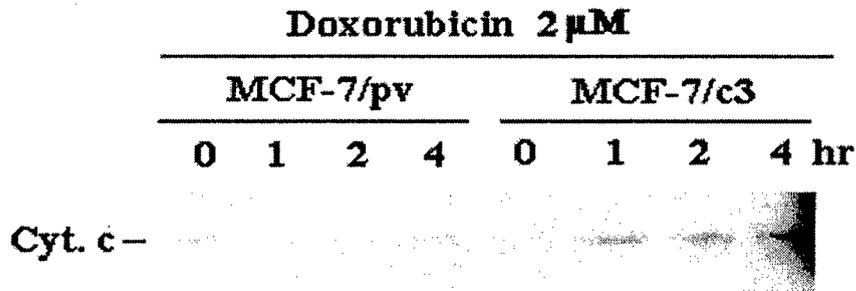


Fig. 15. Feedback action of caspase 3 on cytochrome c release.

MCF-7/pv (caspase deficient) and MCF-7/c (caspase 3 reconstituted) cells were treated with 2 μ M of doxorubicin for times indicated. Fifty μ g of cytosolic extract prepared from the treated cells was separated by SDS-PAGE. Cytochrome c released to cytosol was detected using western blot.

Appendix B

Abstract

Molecular targets and cancer therapeutics, AACR-NCI-EORTC International Conference, Miami, Florida, Oct. 2001

Feedback action of caspase 3 on apical factors, Yang XH, Liu XS, Yang SH, Khandekar JD, Froelich CJ, and Thor AD

Caspases are a group of special proteases involved in the execution of different apoptosis. Activation of caspases is a process of signal transduction mediated by proteolysis, known as caspase cascade. It is believed that activated apical caspases, such as caspases 8 and 9, in turn activate effector caspases, such as caspases 3, 6 and 7, which are responsible for cellular dysfunction and destruction. In contrast to the extensive studies on the activation of effector caspases by apical caspases, the possible role of effector caspases in enhancing apical factor activation is less characterized. In this report, we present our results of the feedback action of caspase 3 on apical factors. To determine the enzymatic kinetics of caspase 3 on apical caspases 2, 8, 9 and 10, in vitro transcription-translated (TNT) products of these apical caspases were treated with purified active caspase 3. Caspase 9 was the preferred substrate of caspase 3 in the pure biochemical system, followed by caspases 2, 8 and 10, suggesting that these apical caspases could be the substrates of caspase 3. To study this feedback action in a whole cell system, we compared caspase 3 reconstituted MCF-7 breast cancer cells (MCF-7/c3) with caspase 3 deficient MCF-7 control cells (MCF-7/pv). We found that activation of caspases 9 and 8, but not caspases 2 and 10, was enhanced in MCF-7/c3 cells after the cells were treated with doxorubicin or TNF- α . The results indicates that caspase 3 indeed has feedback action on caspases 8 and 9. Little difference in caspase 2 activation between MCF-7/pv and MCF-7/c3 cells suggests that other factors might be involved in the feedback process. Caspase 3 mediated feedback action on increased mitochondrial membrane permeability was also detected in MCF-7/c3 cells treated with doxorubicin and ionizing radiation, as shown by increased mitochondrial membrane depolarization and cytochrome c released to cytosol in treated MCF-7/c3 cells. Increased upstream activities detected in MCF-7/c3 cells demonstrated the specific role of caspase 3 in the feedback activation on apical factors. Taken together, these results suggest that caspase 3 could be a potential therapeutic target in apoptosis related diseases.