Indian Journal of Science and Technology http://www.indjst.org Vol.1 No 3 (August 2008)

Differential expression of PML in ^{60}Co γ-ray and γ-IFN- induced apoptosis in B-lymphocytes

Hong Liu[1], Kimio Tanaka[2*] and Nanao Kamada[3]

[1] *Department of Hematology, Affiliated Hospital of Nantong Medical College, Nantong, Jiangsu 226001, China*

[2] *Department of Radiobiology, Institute for Environmental Sciences, Hachazawa 2-121, Takahoko, Rokkasho, Kamikita, Aomori 139 - 3212, Japan*

[3] *Hiroshima Atomic Bomb Relief Foundation, Kurakake, Asakita-ku, Hiroshima 739-1743, Japan*

Email: kmtanaka@ies.or.jp*

Abstract: The function of PML in apoptosis was elucidated in human B lymphocyte- derived cell line. For this purpose, the number of the PML focus and the percentage of apoptotic cells were determined periodically with immunofluorescent staining after ^{60}Coγ-ray or γ-interferons (γ-IFN) treatment. On irradiation with ^{60}Coγ-ray or treatment with γ-IFN, PML protein was over-expressed. This was measured from PML foci, which peaked at 72 hr and 24 hr, respectively. The B-cell line also contained a greater proportion of apoptotic cells after the treatments. The strongest induction of apoptosis both by ^{60}Co-γ ray irradiation and by γ-IFN treatment was observed 24 hr later than the induction of PML expression. In addition, γ- rays-induced apoptosis and PML expression were mediated through caspase-8 but not through caspase-3. However, caspase-8 was involved in γ-IFN-induced PML expression and apoptosis. While caspase-3 is involved solely in PML expression, and partially in apoptosis. These results suggest that ^{60}Co-γ ray irradiation or γ-IFN treatment can induce PML protein expression and apoptosis in the B-cell line as caspase-3 dependently or independently.

Key words: PML Protein, Apoptosis, ^{60}Co-γ ray, γ-IFN, Caspase activation.

Introduction

The promyelocytic leukemia (PML) gene was identified originally as a fusion partner of the RAR alpha gene in the reciprocal chromosomal translocations t(15;17) (q22;q12), specific for acute promyelocytic leukemia (APL) (de The *et al*., 1990, 1991; Goddard *et al*., 1991; Kakizuka *et al*., 1991; Pandolfi *et al*., 1991). PML protein was detected as a band of 90 to 100 KD band on Western blotting when it was expressed after transfection into normal epithelial cells (Daniel *et al*., 1993; Chang *et al*., 1995; Flenghi *et al*., 1995). In addition to having an oncogenic role, PML is essential to several apoptotic pathways and upregulated by stimuli such as irradiation and γ-interferons (γ-IFN) (Chan *et al*., 1997; Chelbi-Alix *et al*., 1995).

The PML protein is associated with nuclear bodies whose functions are as yet unknown. Chan et al found that PML expression was induced 5 to 10 fold at the post-transcriptional level by ionizing radiation or a DNA damaging agents (Chan *et al*., 1997). Chelbi-Alix *et al*. also reported that PML, like Sp100 and NDP52, was induced by γ-IFN in a variety of human cells (Chelbi-Alix *et al*., 1995). However, the role of PML protein in regulating cell growth and apoptosis, and the manner in which PML is expressed after exposure to irradiation and

γ-IFN, are still unclear.

To elucidate the function of PML protein in apoptosis, we measured the number of PML focus and the percentage of apoptotic cells in a B- cell line established from a healthy adult after γ-irradiation or γ-IFN treatment.

The caspase family mediates apoptosis in cells of various origins. Based on their roles in apoptosis, caspases are divided into initiator caspases, including casperses 8, 9 and 10 which are linked to death receptors such as FAS and TNFα, and executioner caspases, including caspases 3, 6 and 7 (Green, 1998) which are activated by a variety of stimuli.

Activation of caspases-3 and -8 is critical in transducing a cascade leading to apoptosis. Current research has advanced our understanding of the role of caspase cascades in the apoptosis induced by various stimuli (Cohen, 1997; Wu & Ding, 2002; He *et al*., 2006; Berg, 1990). Peptide inhibitors specific for caspases are useful for investigating the involvement of caspases in particular apoptotic processes. Additionally, the effects of caspase-3 and -8 inhibitors on PML focus formation and apoptosis in lymphocytes treated with ^{60}Co γ-ray irradiation or with γ-IFN was also examined.

Materials and methods

Preparation of the B-cell line:

The B-cell line T14-1, which was established from the peripheral blood of a healthy adult volunteer by infection with EB-virus in our laboratory, was used for the present study.

Radiation source and lymphocyte culture:

The B-cell line was suspended in RPMI 1640 medium containing 15% fetal calf serum (GIBCO BRL, Gaithersburg, MD) at a final concentration of 1×10^5/ml. The cell suspension was divided and three groups were established: a control group, an irradiated group and a γ-IFN- treated group. Cells of the irradiated group were exposed to 4 Gy of ^{60}Coγ-ray (111 TBq) at a dose rate of 1000 mGy/min at room temperature at the radiation facility of Hiroshima University (Isotron, RTGS-21, Shimadzu Corp. Japan). They were then washed twice with phosphate buffered saline (PBS) and resuspended in RPMI 1640 medium containing 15% fetal calf serum in the presence or absence of the caspase- 3 inhibitor z-DEVD-FMK (100 μM, MBL Co., LTD. , Nagoya, Japan) or the caspase- 8 inhibitor z-IETD-FMK (100 μM, MBL Co., LTD. Nagoya, Japan). These inhibitors were added immediately and the culture was started without stimulation at 37 ^{0}C in 5% CO_2. Cells of the γ-IFN-treated group, in RPMI 1640 medium containing 15% fetal calf serum, were cultured with 1000 U/ml of γ-IFN in the presence or absence of z-DEVD-FMK or z-IETD-FMK. Cells in the control group were set up in a similar way. Samples were collected at 0, 2, 12, 24, 48, 72 and 96 hr. Since the control sample for the irradiation and γ-IFN-exposure experiments yielded similar results, only one control experiment was conducted.

Analysis of PML protein expression by immunofluorescent staining:

Cytospin slides of the three groups of cells were prepared at various time points by centrifugation of 500 rpm for 3 min (Shandon Inc., Pittsburgh, PA, USA) and air dried. The slides were washed in PBS, fixed in methanol for 10 min and

finally immersed in ice-cold acetone for 5 sec to allow the antibodies to penetrate the cells. The fixed slides were blocked with 10% BSA and incubated with PML monoclonal antibody (2 µl/ml; Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 60 min. After three washes with PBS at room temperature, each slide was incubated with biotinylated goat anti-mouse IgG (10 mg/ml; Calbiochem, Cambridge, MA) for 45 min and streptavidin-Texas Red (10 mg/ml; Calbiochem) for 15 min at 37 ^{0}C. Following three more washes with PBS, the prepared slides were mounted in antifade (Oncor, Gaithersburg, MD, USA). Finally, they were scanned under a confocul laser scanning microscope (LSCM) (LSM-GB200; Olympus Optical Co., Tokyo, Japan), and immunofluorescence images were taken and stored in an IBM computer. Analyses of the intensities of signals were performed using application software of LSM-GB200. The 50 cells with PML foci were randomly selected, and the sum of Texas Red-intensities in PML foci of 50 cells were defined as the level of PML expression at each time point.

Quantification of apoptosis:

The B-cell line was quantified for apoptosis using immunofluorescent staining. Briefly, suspensions of B-cells cultured under various conditions were washed with PBS, and then resuspended in 75 µl of annexin V buffer (10 mM Hepes/NaOH pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) containing 1.5 µl of annexin-V-FITC (Boehringer, Mannheim, Germany) and 1.5 µl of propidium iodide (Sigma; 50 µl of 50 µg/ml stock in PBS). After incubation at 37 ^{0}C for 10 to 15 min, cytospin slides were prepared. Apoptotic cells were quantified as annexin-V-positive/propidium iodide-negative cells in 500 cells scored in each slide using immunofluorescence microscopy (Nikon, Tokyo, Japan). Propidium iodide-positive cells were classified into necrosis cells.

Dual color staining analysis of PML expression and apoptosis in identical cell:

Suspensions of B cells prepared under various conditions were washed with PBS then stained with annexin V as previous description. After enumeration of apoptotic cells, the slides were fixed in methanol for 10 min and finally immersed in ice-cold acetone for 5 sec for proper penetration of antibodies. The fixed slides were blocked with 10% BSA and incubated with PML monoclonal antibody. All subsequent procedures were as described before.

Statistical analysis:

On the assumption that the expressions of PML protein or apoptosis distribute randomly at each time point, Poisson distribution analysis was performed.

Results

PML protein expression:

The expression patterns of PML in the B-cells either irradiated with ^{60}Co γ-ray or treated with γ-IFN, are shown in Figure 1. Measurement of the PML protein from the foci by LSCM and immunofluorescent staining revealed an increase in the cells of the irradiated group after only 2 hr incubation. The amount of PML protein peaked at 72 hr, after which it decreased. The expression induced by γ-IFN showed a different pattern

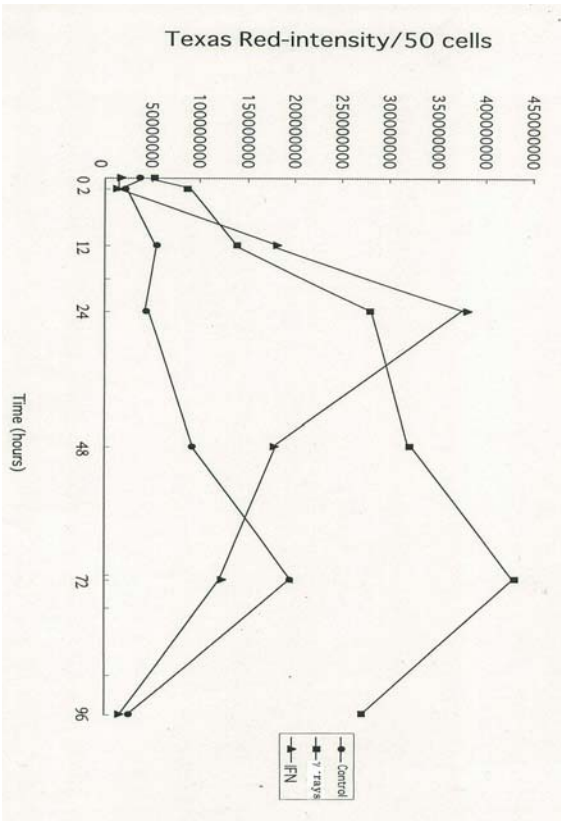
altogether. The peak occurred at 24 hr, and the decrease was sharp with a return to the control level at 96 hr. PML expression in the control group was also slightly increased up until 72 hr. Poisson distribution analysis (Fig.1) showed that expression patterns of γ-IFN- and γ-ray-irradiation-induced PML were different from that of the control ($p<0.01$ and $p<0.01$, respectively). Immunostaining showed that the number of typical PML microspeckles in a lymphocyte increased with prolonged incubation in the control group, and was markedly increased on exposure to ^{60}Coγ-ray- irradiation or treatment with γ-IFN (Fig. 2A, 2B, 3A & 3B). The high intensities of signal indicated increased number of PML microspekle per cell and not larger size of microspekle because the size of the microspeckles was not changed in the control or irradiated group along the culture time (Fig. 2A & 2B).

The number of microspeckles per cell following irradiation was higher at 72 hr than at 24 hr. But the number of PML microspeckles per cell at 24 hr of incubation was greater following γ-IFN treatment than irradiation, and also the size of each microspeckles was smaller after exposure toγ-IFN than irradiation (Fig. 2B & 3B). Most of the PML microspeckles localized to the nucleus in the control and the experimental groups. Increase of PML protein expression correlated with the number of microspeckles per cell.

*Percentages of apoptosis in cells either exposed to radiation or to γ-*IFN-*treatment:*

The percentage of apoptotic cells in the B-cell line was evaluated after treatment with irradiation or γ-IFN by annexin V-staining. As shown in Figure 4, the number of apoptotic cells increased

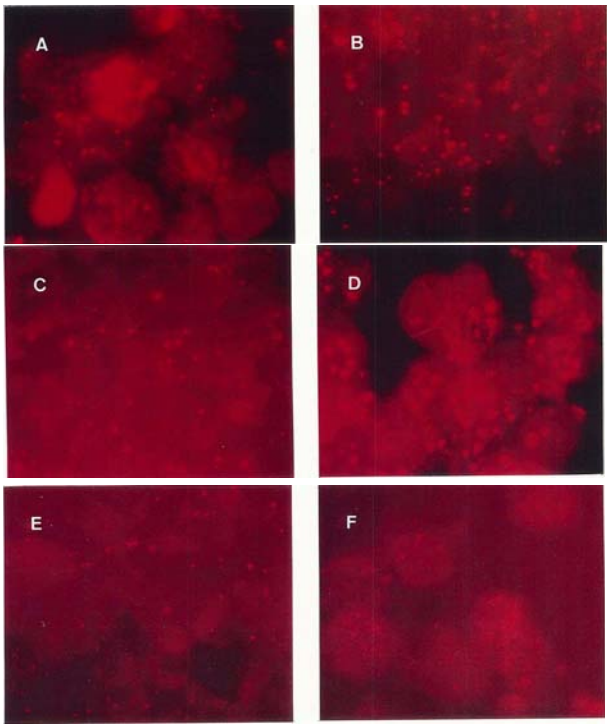
Fig. 1. *PML protein expression in the B-cell line induced by ^{60}Coγ-ray irradiation or treatment with γ-IFN.*



Y-axes shows the sum of Texas Red-intensities in PML foci of 50 cells, which means the level of PML expression. Sum of the Texas-Red intensities of 50 analyzed cells shown at each time point, therefore each point data does not have SD value.

significantly in the irradiated groups, compared with the control. A peak was reached at 96 hr, 24 hr later than the peak of PML expression. Interestingly, a similar phenomenon was observed in cells treated with γ-IFN. The strongest induction of apoptosis by γ-IFN was observed at 48 hr, whereas PML expression peaked at 24 h (Fig. 4). Poisson distribution analysis (Fig.4) showed that expression patterns of γ-IFN- and γ-ray irradiation-induced PML were different from that of

Fig. 2. Immunofluorescence microscopy of PML protein expression induced by ^{60}Coγ-ray irradiation in the presence or absence of caspase-3 or caspase-8 inhibitor.



Cells were harvested after 72 hr culture with or without ^{60}Coγ-ray irradiation and observed by LSCM and immunofluorescent microscope. PML staining is evident as nuclear microspeckles. ***A****: Control sample without ^{60}Coγ-ray irradiation.* ***B****: 72 hr after irradiation.* ***C****: Control sample without irradiation in the presence of caspase-3 inhibitor.* **D**: *72 hr after irradiation in the presence of caspase-3 inhibitor.* ***E****: Control sample without irradiation treatment in the presence of caspase-8 inhibitor.* ***F****: 72 hr irradiation in the presence of caspase-8 inhibitor.*

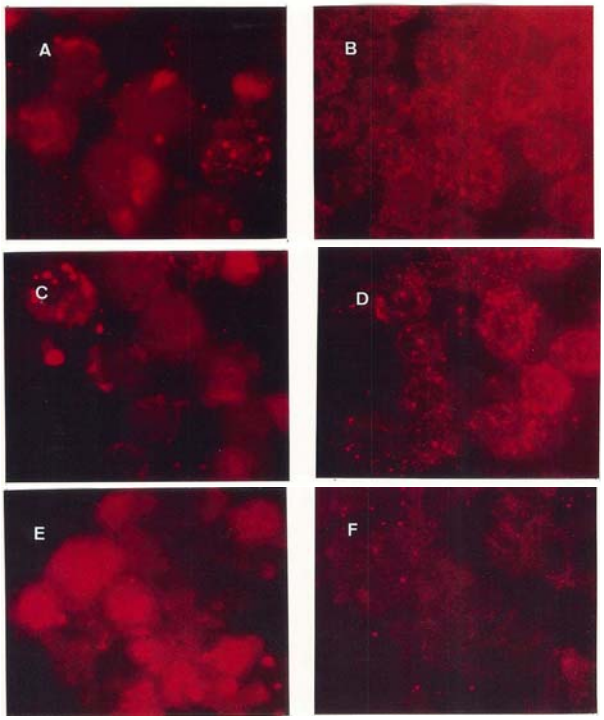
the control ($p<0.01$ and $p<0.01$, respectively). Dual color analysis of PML and annexin-V was used to examine the induction of apoptosis by ^{60}Coγ-ray irradiation and γ-IFN treatment, in which PML was stained red and annexin-V green (Fig. 5 & 6, respectively). The analysis showed that PML-positive cells did not express annexin-V on the cell membrane (Fig. 5F, 6D & 6F). Similarly, apoptotic cells heavily stained with annexin-V did not express PML (Fig. 5F, 6D & 6F). These results indicated that PML protein expression preceded apoptosis.

The effect of caspase-3 and -8 inhibitors on PML expression and apoptosis:

The effect of caspase-3 and -8 inhibitors on the PML expression and apoptosis induced by γ-ray irradiation or γ-IFN treatment in the B-lymphocytes was examined at 72 hr and 24 hr, respectively. Figs. 7A and 7B showed that radiation-induced PML protein expression and apoptosis were dependent not on caspase-3, but on caspase-8. PML protein expression and apoptosis induced by γ-IFN, however, responded differently to both inhibitors (Figs. 8A & 8B) . Statistical analyses were not performed, because these experiments were not repeated. The γ-IFN-induced PML protein expression was inhibited by the caspase-8 inhibitor, z-IETD-FMK (Fig. 2F, 3F, 5F, 6F, 7A, 7B, 8A and 8B), but not by the caspase-3 inhibitor, z-DEVD-FMK (Fig. 2C, 2D, 5C, 5D, 7A, 7B, Fig. 8A & 8B). On comparison, Fig. 7 and Fig. 8 also show that the degree of inhibition varies depending on the inducer (^{60}Coγ-rays or γ-IFN) as well as on the effects on PML expression or induction of apoptosis. Taken together, these results suggest that the apoptosis and PML expression induced by γ-ray irradiation are mediated through caspase-8, but not through caspase-3. However, caspase-8 is involved in the γ-IFN-induced PML expression and apoptosis, while caspase-3 is involved solely in

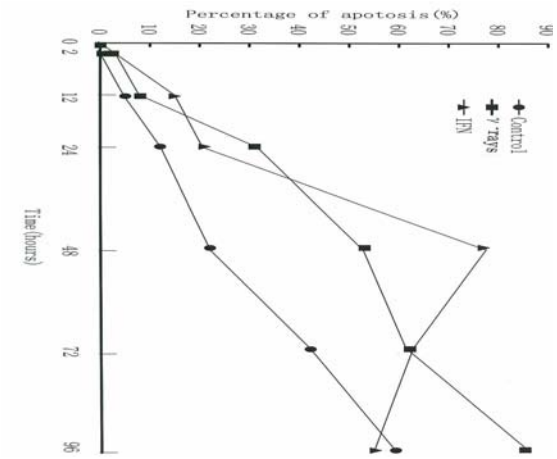
PML expression and partially in apoptosis. To exclude nonspecific effects of these two caspase inhibitors on the lymphocytes, the effects of the inhibitors were examined for PML expression and apoptosis in the presence of the control serum. Neither inhibitors had any significant effect on the lymphocytes (Figs. 2C, 2E, 3C, 3E, 5C, 5E, 6C & 6E).

Fig. 3. *Immunofluorescence microscopy of PML protein expression induced by γ-IFN in the presence or absence of caspase-3 or caspase-8 inhibitor.*



Cells were harvested after 24 hr culture with or without γ-IFN treatment. PML staining is evident as nuclear microspeckles. ***A****: Control sample without γ-IFN treatment.* ***B****: 24 hr after treatment.* ***C****: Control sample without γ-IFN treatment in the presence of caspase-3 inhibitor.* ***D****: Sample after γ-IFN treatment in the presence of caspase-3 inhibitor.* ***E****: Control sample without γ-IFN treatment in the presence of caspase-8 inhibitor.* ***F****: 24 hr after γ-IFN treatment in the presence of caspase-8 inhibitor.*

Fig. 4. *Percentage of apoptotic cells in the B-cell line induced by ^{60}Coy-ray irradiation or γ-IFN.*

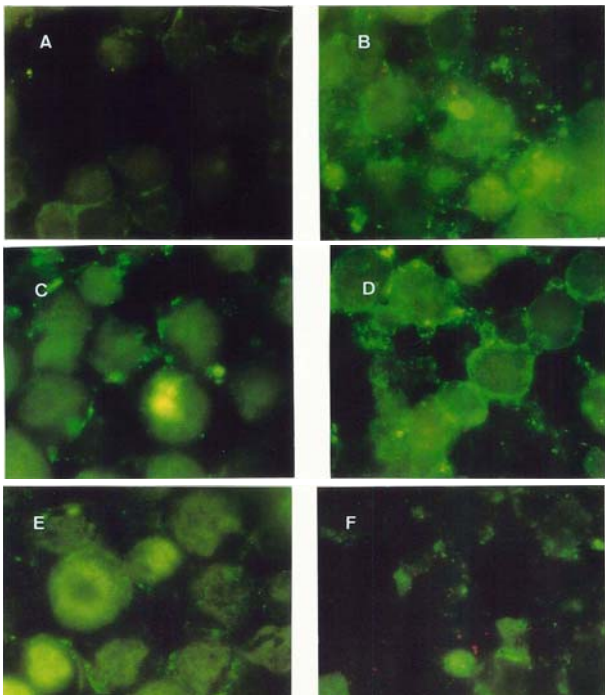


The apoptosis and PML protein expression seem to be interdependent. Comparing the patterns of protein expression (Fig. 1) with those of apoptosis (Fig. 4), the apoptosis occurred 24 hr later than the expression. These data indicated that the expression pattern of PML protein was related with the expression pattern of apoptosis although the possibility that the apoptosis and the expression are independent and the fact could not be excluded. The dual color immunofluorescent analysis of PML and annexin-V on an identical cell undergoing apoptosis after PML expression, will identify the relationship.

Discussion

PML protein belongs to a new family of zinc finger DNA-binding transcription factors (Berg, 1990; Freemont *et al.*, 1991) and exhibits a speckled nuclear localization on immunofluorescence microscopy in APL cells (Daniel *et al.*, 1993; Kastner, *et al.*, 1992). Its function is essential not only for the apoptosis induced by DNA- damage, but by that induced by

Fig. 5. *Immunofluorescence microscopy of apoptosis induced by irradiation in the presence or absence of caspase-3 or caspase-8 inhibitor.*



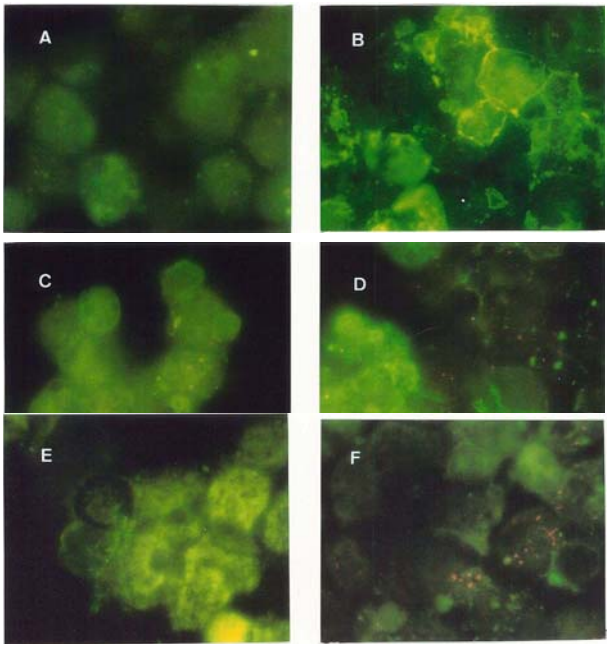
Cells were harvested after 96 hr culture with or without irradiation treatment. AnnexinV-positive cells are shown in green on the membrane. ***A****: Control sample without irradiation treatment.* ***B****: 24 hr after irradiation.* ***C****: Control sample without irradiation in the presence of caspase-3 inhibitor.* ***D****: 24 hr after irradiation in the presence of caspase-3 inhibitor.* ***E****: Control sample without irradiation in the presence of caspase-8 inhibitor.* ***F****: Sample after irradiation in the presence of caspase 8- inhibitor.*

Fas, IFN and tumor necrosis factor (TNF) (Kastner, *et al*., 1992). In the present study, we found that PML protein was over-expressed in a B-cell line after ^{60}Co γ-ray irradiation or γ-IFN treatment. Both treatments also induced apoptosis in this cell line. The percentage of apoptotic cells peaked in the irradiated group at 96 hr and in the γ-IFN -treated group at 48 hr, 24 hr later than the time of PML protein induction. These findings implied that PML protein plays an important preparatory role in the apoptosis, induced both by irradiation and by γ-IFN treatment in B-lymphocytes via different pathways. Although a possibility that apoptosis and PML protein expression is an independent phenomenon could not be excluded. Further analysis of identical cell using immunostaining method with dual color fluorescent will provide clear evidence that the PML expression precedes the apoptosis.

PML protein was induced within 2 hr of irradiation, probably because it expresses associating with p53 transcription and cell cycle arrest (Pearson, 2000). Chan *et al* (1997) suggested that the induction of PML expression is due to radiation-induced DNA damage, which causes arrest at G1. When the damage is too extensive, apoptosis is triggered. In the present study, we demonstrated that radiation-induced apoptosis occurred 24 hr after than PML protein expression. A study showed that PML protein regulates the acetylation of p53 by CBP, and induction of the CDK inhibitor, $p21^{waf1/cip1}$, induced premature senescence by the Ras oncogene (Pearson, 2000). This would indicate that PML protein is an important component of the pathway of signal transduction from p53 to p21, and forms a multiple complex proteins with acetylated p53 and other acetylated proteins (Pearson, 2000). Separate experiments on cell cycling in present T14-1 cell line, showed that the cells were in G2, 48 hr after stimulation with γ-irradiation. The number of apoptotic cells peaked at 96 hr after γ-ray irradiation. Expression times of PML and apoptosis after γ-ray irradiation was consistent with

present result.

Fig. 6. *Immunofluorescence microscopy of apoptosis induced by γ-IFN in the presence or* absence of caspase-3 or caspase-8 inhibitor.

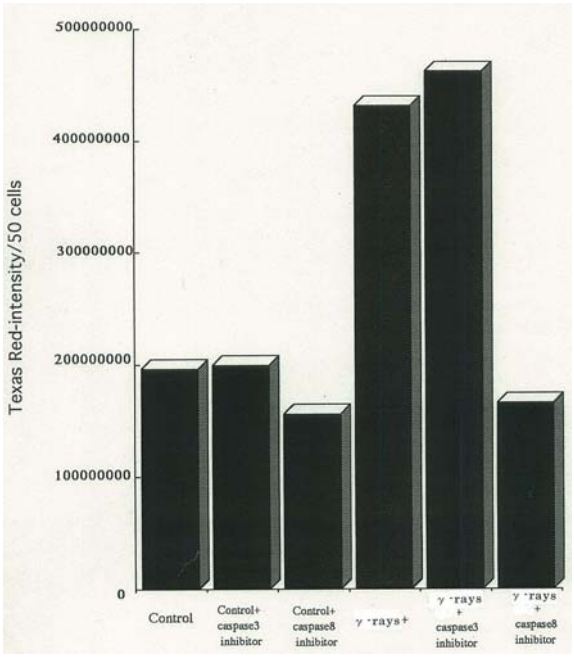


Cells were harvested after 48 hr culture with or without γ-IFN treatment. The percentage of annexin V-positive cells was calculated. Annexin V-positive cells are shown in green. ***A****: Control sample without γ-IFN treatment.* ***B****: 48 hr after γ-IFN treatment.* ***C****: Control sample without γ-IFN treatment in the presence of caspase-3 inhibitor.* ***D****: 48 hr after γ-IFN treatment in the presence of caspase-3 inhibitor.* ***E****: Control sample without treatment in the presence of caspase-8 inhibitor.* ***F****: 48 hr after treatment in the presence of caspase-8 inhibitor. Dual color analysis revealed that cells with PML protein and annexin-V did not show signs of apoptosis, and in turn, apoptotic cells with annexin-V did not express PML. These cells are shown by large or small arrows. PML microspeckles are stained in red and annexin-V in green.*

The γ-IFN is secreted by activated T lymphocytes and natural killer cells. It induces the activation and differentiation of mononuclear phagocytes producing both antiviral and immunomodulatory activities (Chelbi-Alix *et al.*,1995). It has been reported that γ-IFN inhibits hematopoiesis *in vitro*, and induces cell apoptosis (Zoumbos *et al.*, 1984; Maciejewski *et al.*, 1995) and up-regulates PML protein expression (Chelbi-Alix *et al.*, 1995). The present study showed that γ-IFN induces PML expression and apoptosis in a B-cell line. PML protein is induced by both γ-IFN and α/β-IFN (Maciejewski *et al.*, 1995). The protein contains functional α/β-IFN stimulated response elements, ZSRE, and γ-IFN activation sites, GAS (Grotzinger *et al.*, 1996; Stadler et al.,1995; Campbell *et al.*, 1994; Grotzinger *et al.*, 1996), which reveal that PML, like Sp100 and ISG20, is a primary target of IFNs (Chelbi-Alix *et al.*, 1995; Grotzinger *et al.*, 1996; Chelbi-Alix & de The, 1999). The pathway of γ-IFN-induced apoptosis is independent of that of p53 activation (Freemont *et al.*, 1991), like ultraviolet-c-induced apoptosis (Choi *et al.*, 2000), which is different from radiation-induced apoptosis (Ghosh *et al.*, 1999).

Two factors such as γ-rays irradiation and IFN-γ stimulation used for present study were able to influence the subnuclear organization and modified PML expression. Present study revealed that PML protein expression increased correlating with that of the number of microspeckles per cell, but not size of the microspeckles. PML bodies are reported to be contained transcription factors such as Sp100, p53, Rb, HP1 and so on (Pearson, 2000). The differences of the number of

Fig. 7A. *Effect of ^{60}Coγ-ray irradiation on the expression of PML protein in the presence or absence of caspase-3 or caspase-8 inhibitor.*

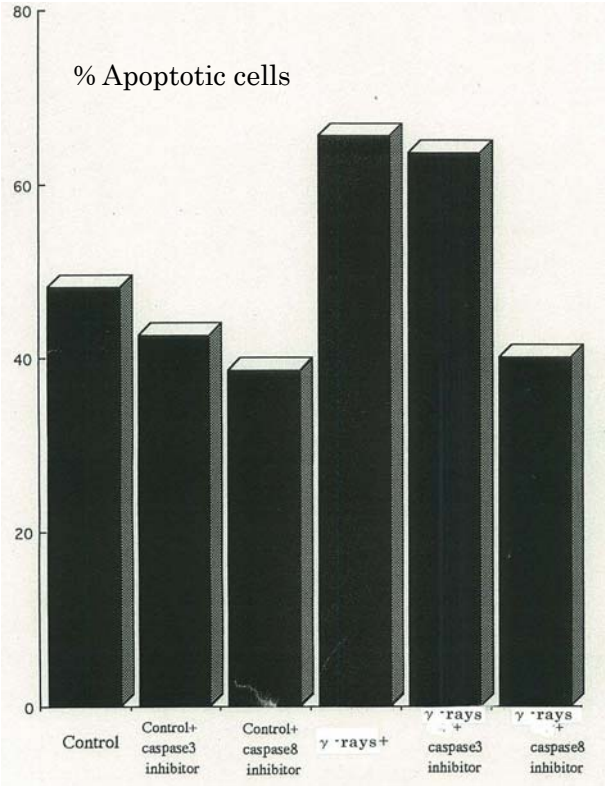


Cells were harvested after 96 hr culture with or without ^{60}Coγ-ray irradiation. Y-axis shows the sum of Texas Red-intensities in PML foci of 50 cells, which means the level of PML expression.

microspeckles per cell or size of the microspeckles among radiation-induced-, IFN-γ stimulating apoptotic cells and non-irradiated cells might be represented quantitative modification of transcriptional factors, meaning an up-regulation of PML protein associated with ongoing apoptosis and so on.

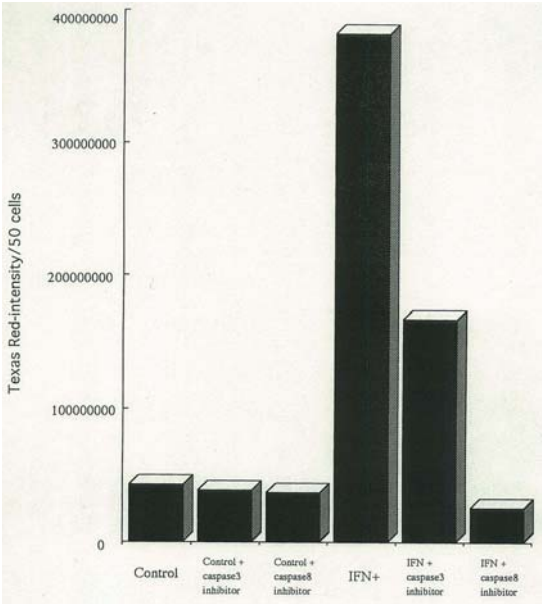
The activated caspases execute the cell death program through degradation of proteins. Caspase-3 is an executioner protease, and appears to have a critical role in apoptosis. It has been shown to be responsible either partially or totally for the proteolytic cleavage of key proteins such as PARP, DNA-PK, actin and U1-70 KD (Green,1998). In the present study, the effect of γ-IFN on the induction of PML expression in B-cells was partially blocked by caspase-3 inhibitor. On the other hand, no significant difference in the extent of expression and apoptosis induced by irradiation was found before and after addition of caspase-3. This finding was in contrast to a report that IFN- and Fas- mediated apoptosis were caspase-3-dependent, and a lack or inhibition of caspase-3 is sufficient to block the apoptosis (Freemont *et al.*, 1991). But it was consistent with Quignon's finding that overexpression of PML induces apoptosis in the absence of caspase-3 activation in rodent cells (Quignon *et al.*, 1998). This phenomenon was not specific for EB

Fig. 7B. *Effect of ^{60}Coγ-ray irradiation on the apoptosis of the B-cells in the presence or absence* of caspase-3 or caspase-8 inhibitor.



Cells were harvested after 96 hr with or without irradiation

Fig. 8A. Effect of γ-IFN on the expression of PML protein in the presence or absence of caspase-3 or caspase-8 inhibitor

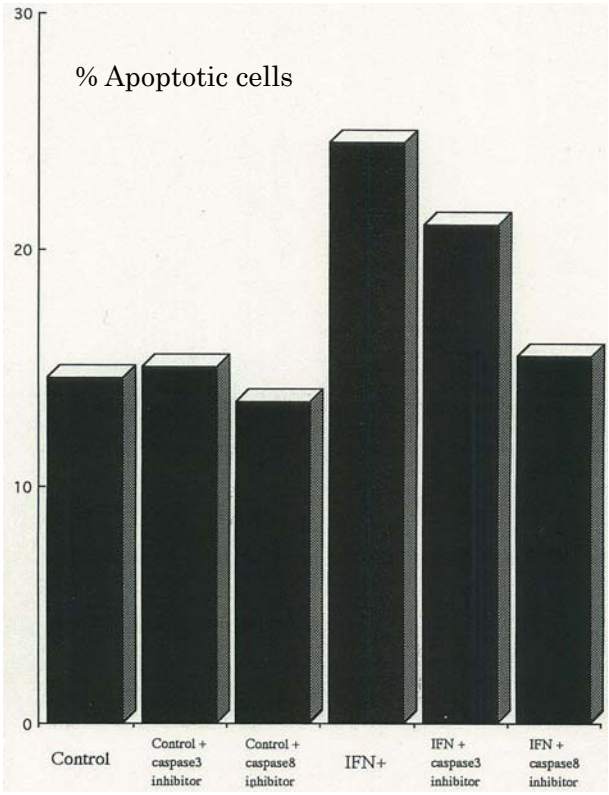


Cells were harvested after 48 hr culture with or without irradiation

virus-stimulated B-cells. It might be that PML protein is involved in the two distinct pro-apoptotic pathways, which are preferentially activated in a cell-type-specific and in a stimulus-specific manner.

Caspase-8 is the first caspase in a cascade of ICE-like proteases activated by FAS (CD95) (Medema et al., 1997). Upon binding to FADD, caspase-8 is converted to its active subunits, which are released into the cytosol and can activate a cascade of ICE-like proteases (Ghosh *et al*.,1999). Hence, caspase-8 is called an initiator protease. Dai *et al*. demonstrated that γ-IFN acts on human erythroid progenitor cells not only to up-regulate Fas, but also to selectively up-regulate caspase 8 and cause apoptosis (Dai & Krantz, 1999). They also found that the apoptosis induced by γ-IFN was

Fig. 8B. Effect of γ-IFN on the apoptosis of the B-cells in the presence or absence of caspase-3 or caspase-8 inhibitor



Cells were harvested after 48 hr culture with or without irradiation

partially blocked in the presence of the caspase-8 inhibitor (Dai & Krantz, 1999). A similar phenomenon was observed in T14-1 B-cell line used in the present study. Our data clearly indicated that caspase-8 inhibitor partly blocks the effect on both PML protein expression and apoptosis of lymphocytes caused by γ-ray irradiation and γ-IFN. This result showed that caspase-8 was activated during the process induced by the two stimuli. The present results also suggested that caspase-8 had an intermediate role in the induction of PML protein expression and apoptosis induced by both ^{60}Co γ-ray irradiation

and γ-IFN in human lymphocytes. Whereas, caspase-3 was not involved especially in radiation-induced apoptosis.

In conclusion, we found that ^{60}Co γ-ray irradiation or γ-IFN treatment can induce PML protein expression and apoptosis in the B-cell line in either caspase-3 dependent or independent manner.

Acknowledgements

We are grateful to Mr. S. Ihda, Department of Radiation Information Center, and to Mr. S. Takeoka, K. Kitagawa and S. Suga of the Radiation Research Center for Frontier Science, Hiroshima University, for assistance. We also thanks to Dr. H. Tanaka of Department of Hematological Oncology, Institute for Radiation Biology and Medicine, for helpful discussion. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports, Japan (K.T.) and by the Natural Science Foundation of Jiangsu, China (No. BK 97149) (H.L.).

References

1. Berg JM (1990) Zinc fingers and other metal-binding domains: elements for interactions between macromolecules. *J. Biol. Chem.* 265, 6513-6516.
2. Campbell IG, Nicolai HM, Foulkes WD, Senger G, Stamp GW, Allan G, Boyer C, Jones K, Bast RC Jr and Solomon E (1994) A novel gene encoding a B-box protein within the BRGA1 region at 17q21.1. *Hums. Mol. Genet.* 3, 589-594.
3. Chan JY, Li L, Fan YH, Mu ZM, Zhang WW and Chang KS (1997) Cell-cycle regulation of DNA damage-induced expression of the suppressor gene PML. *Biochem. Biophys. Res. Commun.* 240, 640-646.
4. Chang KS, Fan YH, Andreeff M, Liu J and Mu ZM (1995) The PML gene encodes a phosphoprotein associated with the nuclear matrix. *Blood* 85, 3646-3653.
5. Chelbi-Alix MK and de The H (1999) Herpes virus induced proteasome-dependent degradation of the nuclear bodies-associated PML and Sp100 protein. *Oncogene* 8, 935-941.
6. Chelbi-Alix MK, Pelicano L, Quignon F, Koken MH, Venturini L, Stadler M, Pavlovic J, Degos L and de The H (1995) Induction of the PML protein by interferons in normal and APL cells. *Leukemia* 9, 2027-2033.
7. Choi KH, Hama-Inaba H, Wang B, Haginoya K, Odaka T, Yamada T, Hayata I, and Ohyama H (2000) UVC- induced apoptosis in human epithelial tumor A431 cells: sequence of apoptotic changes and involvement of caspase (-8 and -3) cascade. *J. Radiat. Res.* 41, 243-258.
8. Cohen GM (1997) Caspases: the executioners of apoptosis. *Biochem. J.* 326, 1-16.
9. Dai CH and Krantz S (1999) Interferon-γ induces upregulation and activation of caspases 1,3, and 8 to produce apoptosis in human erythyroid progenitor cells. *Blood* 93, 3309-3316.
10. Daniel MT, Koken M, Romagne O, Barbey S, Bazarbachi A, Stadler M, Guillemin MC, Degos L, Chomienne C and de The H (1993) PML protein expression in hematopoietic and acute

promyelocytic leukemia cells. *Blood* 82, 1858-1867.

11. de The H, Chomienne, Lanotte M, Degos L and Dejean (1990) The t (15;17) translocation of acute promyelocytic leukemia fuses the retinoic acid receptor alpha gene to a novel transcribed locus. *Nature* 347, 558-684.
12. de The H, Lavau C, Marchio A, Chomienne C, Degos L and Dejean A (1991) The PML-RAR alpha fusion mRNA generated by the t (15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. *Cell* 66, 675-684.
13. Goddard AD, Borrow J, Freemont PS and Solomon E (1991) Characterization of a zinc finger gene disrupted by the t (15;17) in acute promyelocytic leukemia. *Science* 254, 1371-1374.
14. Freemont PS, Hanson IM and Trousdale J (1991) A novel cysteine-rich sequence motif. *Cell* 64, 483-484.
15. Flenghi L, Fagioli M, Tomassoni L, Pileri S, Gambacorta M, Pacini R, Grignani F, Casini T, Ferrucci PF, Martelli MF, Pelicci P G and Falini B (1995) Characterization of a new monoclonal antibody (PG-M3) directed against the aminoterminal portion of the PML gene product: Immunocytochemical evidence for high expression of PML proteins on activated macrophages, endothelial cells, and epithelia. *Blood* 85, 1871-1880.
16. Ghosh CJ, Suzuki K, Kodama S and Watanabe M (1999) Effects of protein kinase inhibitors on the accumulation kinetics of p53 protein in normal human embryo cells following X-irradiation. *J. Radiat. Res.* 40, 23-37.
17. Green DR (1998) Apoptotic pathways: the road to ruin. *Cell* 94, 695-698.
18. Grotzinger T, Jensen K and Will H (1996) The interferon (IFN)-stimulated gene Sp100 promoter contains an IFN-gamma activation site and an imperfect IFN-stimulated response element which mediate type I IFN inducibility. *J. Biol. Chem.* 271, 25253-25260.
19. Grotzinger T, Sternsdorf T, Jensen K and Will H (1996) Interferon-modulated expression of genes encoding the nuclear-dot-associated proteins Sp100 and promyelocytic leukemia protein (PML). *Eur. J. Biochem.* 238, 554-560, 1996.
20. He Y-Y, Huang J-L and Chignell CF (2006) Cleavage of epidermal growth factor receptor by caspase during apoptosis is independent of its internalization. *Oncogene* 25, 1521-1531.
21. Kakizuka, Miller WH Jr, Umesono K, Warrell RP Jr, Frankel SR and Murty VV (1991) Chromosomal translocation t (15;17) in acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML. *Cell* 66,663-674.
22. Kastner P, Perez A, Lutz Y, Rochette EC, Gaub M P, Durand B, Lanotte M, Berger R and Chambon P (1992) Structure localization and transcriptional properties of two classes of retinoic acid receptor alpha fusion proteins in acute promyelocytic leukemia (APL): Structural similarities with a new family of oncoproteins. *EMBO. J.* 11, 629-642.
23. Maciejewski JP, Selleri C, Anderson S and Young NS (1995) Fas antigen expression on

$CD34^+$ human marrow cells is induced by interferon-γ and tumor necrosis factor-α and potentates cytokine-mediated hematopoietic suppression *in vitro*. *Blood* 85, 3183-3190.

24. Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH and Peter ME (1997) FLICE is activated by association with the CD 95 death-inducing signaling complex (DISC). *EMBO J.* 16, 2794-2804.
25. Pandolfi P, Grignani F, Alcalay M, Mencarelli A, Biondi A, LoCoco F, Grignani F, Pelicci PG (1991) Structure and origin of the acute promyelocytic leukemia myl/RAR alpha cDNA and characterization of its retinoid-binding and transactivation properties. *Oncogene* 6, 1285-1292.
26. Pearson M (2000) PML relates p53 acetylation and premature senescence induced by oncogenic Ras. *Nature* 13**,** 207-210.
27. Quignon F, Bels FD, Koken M, Feunteun J, Ameisen JC and de The H (1998) PML induces a novel caspase-independent death process. *Nature Genet.* 20, 259-264.
28. Sen GC and Lengyel P (1992) The interferon system. A bird's eye view of its biochemistry. *J. Biol. Chem.* 267, 5017-5020.
29. Stadler M, Chelbi-Alix MK, Koken MH, Ventvrini L, Lee C, Sab A, Quignon F, Pelicano L, Guillemin MC, Schindler C and de The H (1995) Transcriptional induction of the pml growth suppressor gene by interferons in mediated through ISRE and a GAS element. *Oncogene* 11, 2565-2573.
30. Wang ZG, Ruggero D, Ronchettis S, Zhong S, Gaboli M, Rivi R and Pandolfi PP (1998) Pml is essential for multiple apoptotic pathways. *Nature Genet.* 20, 266-272.
31. Wu GS and Ding Z (2002) Caspase 9 is required for p53-dependent apoptosis and chemosensitivity in a human ovarian cancer cell line. *Oncogene* 21, 1-8.
32. Zoumbos NC, Djeu JY and Young NS (1984) Interferon is the suppressor of hematopoiesis generated by stimulated lymphocytes *in vitro*. *J. Immunol.* 133, 769-774.