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**Contribution of Translin to Hematopoietic Regeneration after Sublethal Ionizing Irradiation**

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Received August 29, 2007; accepted November 29, 2007; published online November 29, 2007

The integrity of the genome is threatened by DNA damaging events such as radiation, viral infection and chemicals. Ionizing irradiation is known to cause genotoxic damage through the generation of reactive oxygen species (ROS) and nitrogen species (RNS) and we have found that a signaling pathway for the nuclear translocation of Translin is initiated in association and efficiently blocked by a specific inhibitor of nitric oxide synthase (NOS). This suggests the involvement of inducible nitric oxide synthase (iNOS)-derived nitric oxide (NO) in the nuclear translocation of Translin. To address the functional significance of Translin in the hematopoietic generation system after ionizing irradiation, we generated Translin-deficient (Translin−/−) mice and examined hematopoietic colony formation after sublethal ionizing irradiation. We thereby confirmed a severe delay of colony formation in the spleens of Translin−/− as compared with Translin+/+ mice. Taken together, the results suggest that Translin contributes to hematopoietic regeneration by acting as a sensor protein for radiation-induced damage.

**Key words** Translin; ionizing irradiation; hematopoietic regeneration

We have previously shown that expression of the octameric ring protein, Translin, closely parallels the proliferative state in various cell types, with protein synthesis starting in S phase and becoming maximal during the G2/M phase. This pattern of periodic expression is most likely associated with functions in the replication of chromosomal DNA or cell division control. In addition, stable transfectant cells expressing inducible Translin under the control of a tetracycline-responsive promoter incorporate BrdU more efficiently than non-expressing cells. This finding suggests that Translin may participate in process ensuring the replication of DNA as well as the acceleration of cell division. Moreover, confocal microscopic analysis has revealed that Translin is localized at the centrosomes at prophase and the mitotic spindle at metaphase, then shifting to midbodies in late telophase. All of these results suggest that Translin participates in processes ensuring the replication of DNA and cytokinesis in mitotic cell division.

In the present investigation, we generated Translin-deficient mice and addressed the question of whether Translin contributes to hematopoietic regeneration after exposure to ionizing irradiation. Evidence was thereby generated that Translin acts like a sensor protein when cells are exposed to various forms of DNA damage such as ionizing irradiation and oxidative stress.

**MATERIALS AND METHODS**

**Nuclear and Cytosolic Preparations** Cells were centrifuged at 1000×g for 5 min and the pellets washed in ice-cold PBS and resuspended in Hypotonic Buffer (10 mM Tris–HCl (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM PMSF). After homogenization with 20 strokes in a Dounce homogenizer, nuclei were pelleted at 2000×g for 10 min, and the supernatant saved as the cytosolic fraction. Nuclear pellets were washed twice, incubated in Rocking Buffer (20 mM Tris–HCl (pH 7.9), 20% glycerol, 300 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 0.5 mM phenyl-methylsulfonyl fluoride (PMSF)) on ice for 30 min and centrifuged at 13000×g to remove any precipitate. Nuclear and cytosolic preparations were precipitated with 10% trichloroacetic acid (TCA), followed by repeated washing with acetone.

**Immunoblotting** For detection of Translin, cell pellets were dissolved in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 5% glycerol, 0.01% bromophenol blue) and run on 10% acrylamide dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) under reducing conditions, transferred to Hybond-polyvinylidene difluoride membranes (Amersham Pharmacia Biotech), and probed with affinity-purified rabbit anti-Translin antibody (1:500) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1500). Antibody binding was detected by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Pharmacia Biotech).

**Generation of Translin-Deficient Mice** The coding sequence of the mouse Translin gene encoding the functional protein is composed of six exons. A gene targeting construct was prepared by deleting an entire exon (10.5 kb). The resulting Translin targeting vector, constructed from a strain 129 library (Stratagene) and consisting of a 3.2 kb homology arm derived from the 5’ end of the exon, a PGK-promoter-neo expression cassette, and a 4.0 kb homology arm from the 3’ end of the exon, was linearized with EcoRI and introduced into GSI ES cells (derived from 129/SvJ) by electroporation. Surviving colonies after selection were picked and expanded for DNA analysis. Targeted ES cells were injected into the blastocoe1 cavity of C57/BL6 embryos using a Piezo-driven mi-
cromanipulator (PrimeTech, Tsuchiura, Japan) to generate chimeric mice which were then bred with C57BL/6 females to obtain heterozygous Translin−/− mutants. These in turn were interbred and found to produce homozygous Translin−/− mice at the expected Mendelian frequency.

**Exposure to Ionizing Irradiation** K562 cells were exposed to 20-Gy dose of irradiation using a 137Cs source, and then Translin levels in both nuclear and cytoplasmic fractions were examined by Western immunoblot analysis. To ask whether Translin contributes to hematopoietic regeneration after ionizing irradiation, Translin+/+ and Translin−/− mice (8—10 weeks) were exposed to a 4-Gy dose of irradiation. At the indicated times, the histological features of hematopoietic regeneration in the spleen were assessed by hematoxylin and eosin staining.

**RESULTS**

Translin Expression Level Is Linked to Cell-Cycle Checkpoint Control in Hematopoietic Cells

One of the most widely used models for hematopoietic differentiation is that featuring PMA (phorbol 12-myristate 13-acetate) treatment of the pluripotent K562 human leukemia cell line.5) This results in irreversible cell cycle arrest and induction of megakaryocytic differentiation in vitro. To determine whether a link between Translin expression and cell proliferation also exists for the hematopoietic system, K562 cells were treated with PMA and the expression levels of Translin protein at various stages were determined by immunoblotting experiments. After exposure to PMA, in accord with cell cycle arrest, the expression levels of Translin protein gradually decreased (Fig. 1A). Since previous studies showed that megakaryocytic differentiation of K562 cells by PMA is induced through the MAPK kinase (MEK)/MAPK pathway,6) we employed a selective inhibitor of the MEK/MAPK pathway, PD98059,7) and established that the PMA-induced inhibition of Translin expression was indeed abrogated (Fig. 1B). A similar result was also obtained with NGF-induced inhibition of Translin expression in PC12 cells (data not shown).

**Ionizing Irradiation and Oxidative Stress Induce Nuclear Translocation of Translin** We previously showed that DNA-damaging reagents, mitomycin C and etoposide initiate a signaling pathway for the nuclear translocation of Translin.8) To test whether Translin is involved in early responses to irradiation, we examined levels in K562 cells within several hours after irradiation. As expected, the majority of Translin was found in the cytoplasm, but a significant amount was also observed in the nucleus after the exposure (Fig. 2A). Nuclear Translin levels reached a peak at around 6h and then returned to the basal levels within 24 h. Although the tumor suppressor gene product p53 also increased in response to ionizing irradiation, nuclear levels continued

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**Fig. 1. Expression of Translin Is Linked to Cell-Cycle Checkpoint Control in Hematopoietic Cells**

(A) After K562 cells were treated with 10 nM PMA, total cell lysates were assessed at the indicated times for Translin levels by immunoblotting. (B) K562 cells were treated with 30 nM PMA for 48 h and total cell lysates were assessed for Translin levels by immunoblotting. The cells were pretreated with PD98059 (20, 50 μM) for 30 min before addition of PMA.

**Fig. 2. Ionizing Irradiation and Oxidative Stress Induces Nuclear Transport of Translin**

(A) K562 cells were exposed to a 20-Gy dose of irradiation, and then Translin levels in both nuclear and cytoplasmic fractions were assessed at the indicated times. (B) As a control, the same samples in (A) were tested for levels of p53. (C) K562 cells pretreated with L-NIO (50 μM) for 15 min were exposed to a 20-Gy dose of irradiation, and then the nuclear Translin levels were assessed at the indicated times by immunoblotting. (D) K562 cells were pretreated with 10 mM H2O2 for 15 min at 37°C, washed with PBS, further incubated, and assessed at the indicated times for nuclear Translin levels by immunoblotting analysis.
to rise after the Translin levels returned to normal (Fig. 2B).

It was shown recently that exposure to ionizing irradiation results in the activation of inducible nitric oxide synthase (iNOS), which generates nitric oxide (NO), a natural mediator involved in a variety of biological processes, including immune responses, neurotransmission, and vasorelaxation.9 Overproduction of NOS has been shown to cause DNA damage and trigger repair processes.10 To ascertain whether NO might be responsible for the nuclear translocation of Translin induced by ionizing radiation, we examined the influence of L-iminoethyl-L-ornithine (L-NIO), a specific inhibitor of iNOS.11 As shown in Fig. 2C, L-NIO reduced the nuclear Translin levels in K562 cells, suggesting the involvement of iNOS-derived NO in the nuclear translocation of Translin during acute radiation responses.

Ionizing irradiation is also known to cause oxidative damage to macromolecules such as proteins, membrane lipids and DNA through the generation of reactive oxygen species (ROS), including H$_2$O$_2$, hydroxyl radicals and superoxide.12,13 H$_2$O$_2$ itself is thought to cause DNA breaks and base modifications through generation of hydroxyl radicals. We therefore tested whether oxidative stress due to H$_2$O$_2$ could induce nuclear translocation of Translin. Surprisingly, the nuclear Translin levels in K562 cells started to increase within 30 min and reached a peak at around 1 to 2 h after the cells were exposed to H$_2$O$_2$, much faster than with ionizing irradiation (Fig. 2D).

**Generation of Translin-Deficient Mice**

To address the functional significance of Translin in the hematopoietic system, we generated mice homozygous for an inactivating mutation of the whole Translin gene. As with its human counterpart, the coding sequence of the mouse Translin gene is assembled from six exons that are spread over a genomic distance of 10.5 kb (Fig. 3A). We prepared a gene targeting construct by deleting entire exons and replacing them with a cassette expressing the neo gene, and the targeted ES clone was then injected into blastocysts to generate chimeric mice. The Translin$^{-/-}$ mice were interbred and found to produce homozygous Translin$^{-/-}$ mice at the expected Mendelian frequency. We confirmed that the wild-type allele is an 8 kb fragment, while the targeted allele is a 5.6 kb fragment by Southern blot hybridization (Fig. 3B). Northern blot analysis demonstrated Translin transcripts to be absent in homozygous mutants (Fig. 3C) and this was confirmed by Western immunoblotting using anti-Translin polyclonal antibodies (Fig. 3D). Translin$^{-/-}$ mice were found to be viable and to have no obvious behavioral abnormalities, while being significantly smaller than their wild-type littermate controls (data not shown).

**Translin Contributes to Hematopoietic Regeneration after Ionizing Irradiation**

In response to ionizing radiation or other environmental stresses, eukaryotic cells are thought to activate signal transduction pathways to arrest cells at specific checkpoints in the cell cycle, to allow repair of damaged DNA.10 To address the functional significance of Translin in the hematopoietic generation system with reference to acute radiation-responses, we examined hematopoietic colony formation in Translin$^{-/-}$ mice after exposure to...
a 4-Gy dose of ionizing irradiation. After 1, 2 and 4 weeks of irradiation, the histological features of extramedullary hematopoiesis in the spleen of Translin\(^{+/+}\) and Translin\(^{-/-}\) mice were assessed by hematoxylin and eosin staining. The results illustrated in Fig. 4 clearly indicate that the hematopoietic colony formation in the spleen of wild mice started 1 week after irradiation and peaked at 2 weeks. However, the same hematopoietic colony formation in the spleens of Translin\(^{-/-}\) mice was delayed more than 2 weeks compared with Translin\(^{+/+}\) mice.

**DISCUSSION**

We have demonstrated that PMA treatment of K562 cells induced inhibition of Translin expression in accord with cell cycle arrest. Moreover, a selective inhibitor of MEK/MAPK pathway, PD98059, abrogated the PMA-induced inhibition of Translin expression. Given the involvement of the MEK/MAPK pathway in growth arrest with concomitant induction of p21\(^{WA F1/CIP1}\), a potent inhibitor of cyclin-dependent kinase (CDK) activity,\(^{14}\) it is conceivable that Translin gene expression is down-regulated by cell cycle arrest, accompanied by induction of CDK inhibitory proteins.

In a previous report,\(^{4}\) we showed that expression of Translin is associated with cell cycle checkpoint defects in lymphoid cells from cases of Ataxia-telangiectasia (AT), a recessive human genetic disorder resulting from mutations of the Atm gene,\(^{15}\) characterized by progressive neuro-degeneration, immunologic defects, cancer predisposition, and hypersensitivity to ionizing irradiation.\(^{16,17}\) While DNA damage responses after ionizing irradiation normally cause cell cycle arrest to allow cells to carry out DNA repair, AT cells show an irradiation-induced cell cycle checkpoint defect.\(^{18}\) In our data, cell cycle checkpoint defects of AT cells are associated with altered expression of the Translin protein, providing further support for a general tight link with cell proliferation in acute radiation responses.\(^{4}\) In addition, we found that the nuclear translocation of Translin is induced by ionizing irradiation and efficiently blocked by a specific inhibitor of iNOS. This suggests the involvement of iNOS-derived NO in the nuclear translocation of Translin during acute radiation responses.

The free radical generator H\(_2\)O\(_2\) has been found to induce tyrosine phosphorylation of intracellular proteins.\(^{19—21}\) In this regard, recent studies have demonstrated that H\(_2\)O\(_2\) promotes tyrosine phosphorylation and rapid nuclear translocation of STAT3.\(^{22}\) We found that the nuclear Translin levels reached a peak at around 1 to 2 h after the cells were exposed to H\(_2\)O\(_2\), much faster than with ionizing irradiation. While the precise molecular mechanism remains unclear, it may be speculated that exogenously added high dose of H\(_2\)O\(_2\) (10 mM) could induce rapid nuclear translocation of Translin by phosphorylation.

To address the functional significance of Translin in the hematopoietic generation system after ionizing irradiation, we generated Translin-deficient mice and examined hematopoietic colony formation after sublethal ionizing irradiation. We confirmed a severe delay of colony formation in the spleens of Translin\(^{-/-}\) compared with Translin\(^{+/+}\) mice, clearly indicating that Translin contributes the hematopoietic colony formation for radiation-induced damage. Although a number of potential molecules linked with stress responses and altered cell cycle regulation have been identified,\(^{23}\) we have shown that Translin is involved in the mechanism by which hematopoietic cells regenerate after exposure to ionizing irradiation. Thus, the present results point to opportunities for translating research findings into clinical application in the recovery from radiation-induced injury.

**Acknowledgment** This work was supported by the Budget for Nuclear Research of the MEXT, awarded to M.K.

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**Fig. 4. Translin Deficiency Results in Delayed Hematopoietic Regeneration after Ionizing Irradiation**

Translin\(^{+/+}\) and Translin\(^{-/-}\) mice were exposed to a 4-Gy dose of irradiation. At the indicated times, the histological features of extramedullary hematopoiesis in the spleen were assessed by hematoxylin and eosin staining.
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