Erratum to “Impairment in S-phase entry of splenocytes of Parp-1 knockout mice”
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In this paper, the phrase should be corrected as follows:

(page 249, right column, line 10)
For “with 50 µM ionomycin/ 1 µM phorbol”
read “with 0.5 µM ionomycin/ 10 nM phorbol”.

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Impairment in S-phase entry of splenocytes of Parp-1 knockout mice

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Abstract: One immediate cellular response to DNA damage is the polyADP-ribosylation reaction by poly(ADP-ribose) polymerase-1 (Parp-1). The importance of Parp-1 has been established in many cellular processes, such as the maintenance of genomic stability, DNA repair and cell-death induction. Here, we established Parp-1−/− mice of C57BL/6J congenic strain and characterized the role of Parp-1 in cell-cycle progression. In this study, we also improved a method to observe G0/G1 to S-phase transition of splenocytes and bone marrow cells prepared from mice. The cells were cultured and stimulated with mitogens (50 μM ionomycin/1 μM phorbol 12, 13-dibutyrate). We found that addition of a commercially available growth supportive reagent, BM Condimed R H1, greatly enhanced the transition of G0/G1 to the S-phase, which was determined by bromodeoxyuridine (BrdU) incorporation to DNA. Using this method, G0/G1 to the S-phase entry was measured using splenocytes derived from Parp-1−/−, Parp-1+/− and wild-type (Parp-1+/+) mice. DNA synthesis in Parp-1+/+ and Parp-1+/− splenocytes started from day 1 after addition of mitogens, whereas that in Parp-1+/− cells started from day 2. The peak of the S-phase was at day 2 in all genotypes and notably DNA synthesis in Parp-1−/− cells was approximately halved compared to Parp-1+/+ cells on day 2, 3 and 4. These results suggested that Parp-1 is involved in positive regulation of S-phase entry in quiescent mouse splenocytes.

Key words: Poly (ADP-ribose) polymerase; cell-cycle; splenocytes; S-phase; 3-amino-benzamide; BM Condimed R H1.

Introduction. Protein polyADP-ribosylation is one post-translational modification on various nuclear and centrosomal proteins.1,2) The synthesis of poly(ADP-ribose) from β-NAD+ in response to DNA strand breaks is mostly catalyzed by Parp-1,1,2) which is localized in nuclei and centrosomes.3-5) In vitro and in vivo experimental studies, including gene-disruption studies, indicate Parp-1 as an active participant in regulation of genomic stability,6,7) DNA repair,8) gene expression9,10) as well as in cellular differentiation11,12) and cell-death induction.13) Furthermore, involvement of Parp-1 on DNA replication and cell cycle regulation, including G0/G1 to S-phase transition,14) G1 and G2 arrest after DNA damages15,16) were also suggested. On the other hand, another DNA strand break-sensing molecule, the DNA-PKcs-Ku70/80 complex (DNA-PK complex), is also involved in the regulation of cell-cycle progression17) as well as in DNA strand break repair. Recently, we reported the involvement of DNA-PK complex in the control of cell-cycle progression at G1 to S-phase transition by affecting the activity of a transcription factor, E2F.18) Therefore, both Parp-1 and DNA-PK complex, which directly recognize DNA damage, are involved in the cell-cycle checkpoint control. Furthermore, the coordinative interaction between Parp-1 and DNA-PK complex is reported.19,20) Parp-1 stimulates DNA-PK activity19) whereas DNA-PK inhibits...
To understand the function of the Parp-1 as well as DNA-PK complex on cell-cycle progression, various experimental systems need to be used because the function of these proteins might be different among tissues and cell types. In the present study, we investigated the S-phase entry of quiescent splenocytes of Parp-1-/- mice. We unexpectedly succeeded in a marked improvement of the stimulation of S-phase entry in splenocytes and bone marrow cells using BM Condimed RH1. Using this modified method, we demonstrated that Parp-1 deficiency negatively affects G0/G1 to S-phase transition.

**Materials and methods.** Preparation and culture conditions of splenocytes, bone marrow cells and thymocytes. All animals were maintained in a room controlled for 12 hrs light/dark cycle, temperature (25°C), and humidity (50 ± 10%). Parp-1-/- mice harbor exon 1 disruption through the insertion of a neomycin resistance gene cassette.25) Parp-1-/- congenic mice of C57BL/6J genetic background were established by backcrossing the mice of ICR/129Sv mixed genetic background for 8 successive generations with C57BL/6J mice. Splenocytes, bone marrow cells and thymocytes were isolated from 5-week-old male C57BL/6J mice as previously described25) and plated at a density of 1×10⁵ cells in 0.1-ml culture into 96-well plates for the experiment in Fig. 1. Splenocytes of Parp-1-/-, Parp-1+/- and wild-type (Parp-1+/-) male mice were isolated at 12-week of age and plated as above for the experiment in Fig. 2. These cells were cultured in RPMI 1640 supplemented with 10 % fetal bovine serum and BM Condimed H1 (Boehringer Mannheim) and were stimulated with 0.5 µM ionomycin/ 10 nM phorbol 12, 13-dibutyrate for 1, 2, 3 and 4 days22) in a triplicate manner. It is described that BM Condimed H1 is prepared from the supernatant of a mouse thymoma cell line stimulated with 12-O-tetradecanoylphorbol 13-acetate and contains a complex mixture of growth factors and cytokines.23) It contains 15% fetal calf serum, 1 mM oxalacetate, 1 mM sodium pyruvate, 0.2 mg/ml insulin, 1 ng/ml human interleukin 6 and 10 ng/ml 12-O-tetradecanoylphorbol 13-acetate.

Measurement of DNA synthesis in splenocytes, bone marrow cells and thymocytes. Bromodeoxyuridine (BrdU) was added into culture medium 2 hrs before harvesting cells. BrdU incorporation into DNA was measured subsequently by enzyme immunoassay using
an ELISA kit (Boehringer Mannheim) as previously described.24)

Results and discussion. We established Parp-1\(^{+/+}\) congenic mice of the C57BL/6J strain. Brother-sister matings of Parp-1\(^{+/-}\) mice produced offspring at the ratio of 4 (Parp-1\(^{++}\)):5 (Parp-1\(^{-/-}\)):1 (Parp-1\(^{-/-}\)) (total number of offspring was 60). Thus a decreased ratio of Parp-1\(^{-/-}\) offspring than Mendelian ratio was observed, although Parp-1\(^{-/-}\) mice were fertile. When E13.5 embryos obtained from brother-sister mating of Parp-1\(^{+/-}\) mice were genotyped, Parp-1\(^{-/-}\) embryos (6/21) were observed in a Mendelian fashion, suggesting that Parp-1 deficiency could possibly affect the embryogenesis later than this stage.

To investigate \textit{de novo} DNA synthesis in mitogen-stimulated cells using ELISA, we isolated splenocytes, bone marrow cells and thymocytes from wild-type mice. When these cells were exposed to the mitogens, 50 µM ionomycin/1 µM phorbol 12, 13-dibutyrate, in the absence of BM Condimed \(^{8}\)H1, the DNA synthesis measured by BrdU incorporation was hardly detectable. However, addition of BM Condimed \(^{8}\)H1 increased DNA synthesis 85-fold in splenocytes and 31-fold in bone marrow cells. On the other hand, DNA synthesis of thymocytes was only augmented 8-fold (Fig. 1A and 1B). Because BM Condimed \(^{8}\)H1 contains specific growth factors for B cells,\(^{25}\) it is possible that the B-cell population in the splenocytes and bone marrow cells was influenced but the T-cell population, which is the main component of thymocytes, was not affected. These results indicated that BM Condimed \(^{8}\)H1 is useful for detection of \textit{de novo} DNA synthesis in mitogen-stimulated splenocytes and bone marrow cells.

Using this novel modified method, we measured DNA synthesis of splenocytes from Parp-1\(^{++}\), Parp-1\(^{-/-}\) and Parp-1\(^{-/-}\) mice after mitogen stimulation, as shown in Fig. 2. DNA synthesis in Parp-1\(^{++}\) and Parp-1\(^{-/-}\) splenocytes was detected from day 1 after mitogen stimulation, whereas that in Parp-1\(^{-/-}\) splenocytes started at day 2 after mitogen stimulation. DNA synthesis peaked at day 2 in all genotypes, however, DNA synthesis in Parp-1\(^{-/-}\) splenocytes was almost halved compared to that in Parp-1\(^{++}\) cases on day 2, 3 and 4. It is notable that Parp-1\(^{-/-}\) splenocytes also showed a lowered level of DNA synthesis at day 3 and 4, as in Parp-1\(^{-/-}\) splenocytes. These results led to the conclusion that Parp-1 is involved in the regulation of the cell cycle when quiescent splenocytes at G0/G1-phase enter the S-phase. Since the length of the transition time from G0/G1 to the S-phase was not influenced in Parp-1\(^{-/-}\) animals compared to Parp-1\(^{++}\) and Parp-1\(^{-/-}\) animals, it is suggested that Parp-1 deficiency lowered the frequency of S-phase entry in splenocytes after mitogen-stimulation, but did not alter the sequential cascade for S-phase entry itself. It is also possible that process of DNA synthesis in S-phase in Parp-1\(^{-/-}\) splenocytes may be slower than in Parp-1\(^{++}\) cells.

Our findings are consistent with the result reported by Rosenthal \textit{et al.} on mouse embryonic fibroblasts.\(^{15}\) They reported that G0/G1 to S-phase entry in serum-starved cells is decreased and that the expression of necessary genes in G1 to S-phase transition depending on E2F-1 promoter activity is lowered in Parp-1\(^{-/-}\) embryonic fibroblasts.
In addition, we also investigated the effect of a polyADP-ribosylation inhibitor 3-aminobenzamide (3-AB) on de novo DNA synthesis in wild-type (Parp-1+/+) splenocytes. DNA synthesis in the splenocytes after mitogen stimulation in the presence or the absence of 4 mM 3AB was 0.20 ± 0.045 and 0.15 ± 0.036 Abs450, respectively, and no significant difference was observed. Thus, inhibition of Parp-1 activity did not result in the impairment of DNA synthesis. This suggests the presence of Parp-1 protein itself, but not polyADP-ribosylation, is necessary for G0/G1 to S-phase entry of splenocytes. Since Parp-1 is known to interact with various proteins involved in cell-cycle progression through the BRCT (BRCA-1 C-terminus) domain, the protein-protein interaction may be important for the stimulating effect of Parp-1 on G0/G1 to S-phase transition.

The improved method to detect S-phase entry of splenocytes and bone marrow cells should be useful for further characterization of the role of Parp-1 as well as other regulatory molecules in the process of G0/G1 to S-phase transition.

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References

1) de Murcia, G., and Meissier-de Murcia, J. (1994) Trends Biochem. Sci. 19, 172-176.
2) D’Amours, D., Desnoyers, S., D’Silva, I., and Poirier, G. G. (1999) Biochem. J. 342, 249-268.
3) Yamanaka, H., Penning, C. A., Willis, E. H., Wasson, D. B., and Carson, D. A. (1988) J. Biol. Chem. 263, 3879-3883.
4) Ludwig, A., Behnke, B., Holthund, J., and Hilz, H. (1988) J. Biol. Chem. 263, 6993-6999.
5) Kanai, M., Uchida, M., Hanai, S., Uematsu, N., Uchida, K., and Mizu, M. (2000) Biochem. Biophys. Res. Commun. 278, 385-389.
6) Meissier-de Murcia, J., Niedergang, C., Trucco, C., Ricoul, M., Dutrillaux, B., Mark, M., Oliver, F. J., Masson, M., Dierich, A., LeMeur, M., Watzinger, C., Chambon, P., and de Murcia, G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7303-7307.
7) Wang, Z. Q., Stingl, L., Morrison, C., Jantsch, M., Los, M., Schulze-Osthoff, K., and Wagner, E. F. (1997) Genes Dev. 11, 2347-2358.
8) Dantzer, F., Schreiber, V., Niedergang, C., Trucco, C., Flatter, E., de La Rubia, G., Oliver, J., Rolli, V., Meissier-de Murcia, J., and de Murcia, G. (1999) Biochemie 81, 69-75.
9) Simbulan-Rosenthal, C. M., Ly, D. H., Rosenthal, D. S., Konopka, G., Luo, R., Wang, Z. Q., Schultz, P. G., and Smulson, M. E. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 11274-11279.
10) Hassa, P. O., and Hottiger, M. O. (1999) Biol. Chem. 380, 953-959.
11) Nozaki, T., Masutani, M., Watanabe, M., Ochiya, T., Hasagawa, F., Nakagama, H., Suzuki, H., and Sugimura, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13345-13350.
12) Hemberger, M., Nozaki, T., Winterhager, E., Yamamoto, H., Nakagama, H., Kamada, N., Suzuki, H., Ohta, T., Oishi, M., Masutani, M., and Cross, J. C. (2003) Dev. Biol. 257, 371-381.
13) Smulson, M. E., Kang, V. H., Niambi, J. M., Rosenthal, D. S., Ding, R., and Simbulan, C. M. G. (1996) J. Biol. Chem. 270, 119-127.
14) Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994) Nature 371, 346-347.
15) Simbulan-Rosenthal, C. M., Rosenthal, D. S., Luo, R., and Smulson, M. E. (1999) Oncogene 18, 5015-5023.
16) Wieler, S., Gagne, J. P., Vaziri, H., Poirier, G. G., and Benchimol, S. (2003) J. Biol. Chem. 278, 18914-18921.
17) Nozaki, T., Masutani, M., Akagawa, T., Sugimura, T., and Esami, H. (1994) Jpn. Cancer Res. 85, 1094-1098.
18) Watanabe, F., Shinohara, K., Teraoka, H., Komatsu, K., Tatsuni, K., Suzuki, F., Imai, T., Sagara, M., Tsuji, H., and Ogiu, T. (2003) Intern. J. Biochem. Cell Biol. 35, 432-440.
19) Ruscetti, T., Lehner, B. E., Halbrook, J., Trong, H. L., Hoekstra, M. F., Chen, D. J., and Peterson, S. R. (1998) J. Biol. Chem. 273, 14461-14467.
20) Ariumi, Y., Masutani, M., Copeland, T. D., Mimori, T., Sugimura, T., Shimotohno, K., Ueda, K., Hatanaka, M., and Noda, M. (1999) Oncogene 18, 4616-4625.
21) Masutani, M., Suzuki, H., Kamada, N., Watanabe, M., Ueda, O., Nozaki, T., Jishage, K., Watanabe, T., Sugimoto, T., Nakagama, H., Ochiya, T., and Sugimura, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2301-2304.
22) Nagaawa, M., Watanabe, F., Suwa, A., Yamamoto, K., Tsukada, K., and Teraoka, H. (1997) Cell Struct. Funct. 6, 585-594.
23) Stein, G. H., and St Clair, J. A. (1988) In Vitro Cell Dev. Biol. 5, 381-387.
24) Zwerger, T., Kakirman, H., Rohde, V., Wallich, B., and Unterregger, G. (1998) Eur. Urol. 33, 414-423.
25) Bork, P., Hofmann, K., Bucher, P., Neuwald, A. F., Altschul, S. F., and Koonin, E. V. (1997) PASEB J. 11, 68-76.