REFERENCES

1. Wynman, C. and Kanaar, R. (2006) DNA double-strand break repair: All's well that ends well. Ann. Rev. Genet. **40**: 363–383.
2. Lavin, M. F. and Kozlov, S. (2007) ATM activation and DNA damage response. Cell Cycle **6**: 931–942.
3 Zhou, B-B. S. and Elledge, S. J. (2000) The DNA damage response: putting checkpoints in perspective. Nature **408**: 433–439.
4. Shiloh, Y. (2003) ATM and related protein kinases: safeguarding genome integrity. Nat. Rev. Cancer **3**: 155–168.
5. Iwabuchi, K., Bartel, P. L., Li, B., Marraccino, R. and Fields, S. (1994) Two cellular proteins that bind to wild-type but not mutant p53. Proc. Natl. Acad. Sci. USA. **91**: 6098–6102.
6. Iwabuchi, K., Li, B., Massa, H. F., Trask, B. J., Date, T. and S. Fields, S. (1998) Stimulation of p53-mediated transcriptional activation by the p53-binding proteins, 53BP1 and 53BP2. J. Biol. Chem. **273**: 26061–26068.
7. Joo, W. S., Jeffrey, P. D., Cantor, S. B., Finnin, M. S., Livingston, D. M. and Pavletich N. P. (2002) Structure of the 53BP1 BRCT region bound to p53 and its comparison to the Brca1 BRCT structure. Genes Dev. **16**: 583–593.
8. Derbyshire, D. J., Basu, B. P., Serpell, L. C., Joo, W. S., Date, T., Iwabuchi, K. and Doherty, A. J. (2002) Crystal structure of human 53BP1 BRCT domains bound to p53 tumour suppressor. EMBO J. **21**: 3863–3872.
9. Schultz, L. B., Chehab, N. H., Malikzay, A. and Halazonetis, T. D. (2000) p53-binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks. J. Cell Biol. **151**: 1381–1390.
10. Rappold, I., Iwabuchi, K., Date, T. and Chen, J. (2001) Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways. J. Cell Biol. **153**: 613–620.
11. Charier, G., Couprie, J., Alpha-Bazin, B., Meyer, V., Quéméneur, E., Guérois, R., Callebaut, I., Gilquin, B. and Zinn-Justin, S. (2004) The Tudor tandem of 53BP1: a new structural motif involved in DNA and RG-rich peptide binding. Structure **12**:1551–1562.
12. Huyen, Y., Zgheib, O., Ditullio, R. A. Jr., Gorgoulis, V. G., Zacharatos, P., Petty, T. J., Sheston, E. A., Mellert, H. S., Stavridi, E. S. and Halazonetis, T. D. (2004) Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks. Nature **432**: 406–411.
13. Sanders, S. L., Portoso, M., Mata, J., Bähler, J., Allshire, R. C. and Kouzarides, T. (2004) Methylation of histone H4 lysine 20 controls recruitment of Crb2 to sites of DNA damage. Cell **119**: 603–614.
14. Xia, Z., Morales, J. C., Dunphy, W. G. and Carpenter, P. B. (2000) Negative cell cycle regulation and DNA-damage inducible phosphorylation of the BRCT protein 53BP1. J. Biol. Chem. **276**: 2708–2718.
15. Anderson, L., Henderson, C. and Adachi, Y. (2001) Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage. Mol. Cell. Biol. **21**: 1719–1729.
16. Wang, B., Matsuoka, S., Carpenter, P. B. and Elledge, S. J. (2002) 53BP1, a mediator of the DNA damage checkpoint. Science **298**: 1435–1438.
17. DiTullio, R. A., Mochan, T. A., Venere, M., Barrtkova, J., Sehested, M., Bartek, J. and Halazonetis, T. D. (2002) 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. Nat. Cell Biol. **12**: 998–1002.
18. Fernandez-Capetillo, O., Chen, H. T., Celeste, A., Ward, I., Romanienko, P. J., Morales, J. C., Naka, K., Xia, Z., Camerini-Otero, R. D., Motoyama, N., Carpenter, P. B., Bonner, W. M., Chen, J. and Nussenzweig, A. (2002) DNA damage-induced G2/M checkpoint activation by histone H2AX and 53BP1. Nat. Cell Biol. **12**: 993–997.
19. Iwabuchi, K., Piku-Basu, B., Kysela, B., Kurihara, T., Shibata, M., Guan, D., Cao, Y., Hamada, T., Imamura, K., Jeggo, P. A., Date, T. and Doherty, A. J. (2003) Potential role for 53BP1 in DNA end-joining repair through direct interaction with DNA. J. Biol. Chem. **278**: 36487–36495.
20. Jackson, S. P. (2002) Sensing and repairing DNA double-strand breaks. Carcinogenesis **23**: 687–696.
21. Jeggo, P. A. (1998) DNA breakage and repair. Adv. Genet. **38**: 185–211.
22. Rothkamm, K., Kruger, I., Thompson, L. H. and Lobrich, M. (2003) Pathways of DNA double-strand break repair during the mammalian cell cycle. Mol. Cell. Biol. **23**: 5706–5715.
23. Riballo, E., Kuhne, M., Rief, N., Doherty, A., Smith, G. C., Recio, M. J., Reis, C., Dahm, K., Fricke, A., Krempler, A., Parker, A. R., Jackson, S. P., Gennery, A., Jeggo, P. A. and Löbrich, M. (2004) A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to γ-H2AX foci. Mol. Cell **16**: 715–724.
24. Iwabuchi, K., Hashimoto, M., Matsui, T., Kurihara, T., Shimizu, H., Adachi, N., Ishiai, M., Yamamoto, K., Tauchi, H., Takata, M., Koyama, H. and Date, T. (2006) 53BP1 contributes to survival of cells irradiated with X-ray during G1 without Ku70 or Artemis. Genes Cells **11**: 935–948.
25. Manis, J. P., Morales, J. C., Xia, Z., Kutok, J. L., Alt, F. W. and Carpenter, P. B. (2004) 53BP1 links DNA damage-response pathway to immunoglobulin heavy chain class-switch recombination. Nat. Immunol. **5**: 481–487.
26. Ward, I. M., Reina-San-Martin, B., Olaru, A., Minn, K., Tamada, K., Lau, J. S., Cascalho, M., Chen, L., Nussenzweig, A., Livak, F., Nussenzweig, M. C. and Chen, J. (2004) 53BP1 is required for class switch recombination. J. Cell Biol. **165**: 459–464.
27. Petersen, S., Casellas, R., Reina-San-Martin, B., Chen, H. T., Difilippantonio, M. J., Wilson, P. C., Hanitsch, L., Celeste, A., Muramatsu, M., Pilch, D. R., Redon, C., Ried, T., Bonner, W. M., Honjo, T., Nussenzweig, M. C. and Nussenzweig, A. (2001) AID is required to initiate Nbs1/γ-H2AX focus formation and mutations at sites of class switching. Nature **414**: 660–665.
28. Kobayashi, J., Antoccia, A., Tauchi, H., Matsuura, S., Komatsu, K. (2004) NBS1 and its functional role in the DNA damage response. DNA Repair **3**: 855–861.
29. Bakkenist, C. J. and Kastan, M. B. (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature **421**: 499–506.
30. Tauchi, H., Kobayashi, J., Morishima, K., van Gent, D. C.,

Shiraishi, T., Verkaik, N. S., vanHeems, D., Ito, E., Nakamura, A., Sonoda, E., Takata, M., Takeda, S., Matsuura, S. and Komatsu, K. (2002) Nbs1 is essential for DNA repair by homologous recombination in higher vertebrate cells. Nature **420**: 93–98.

31. Kobayashi, J., Tauchi, H., Sakamoto, S., Nakamura, A., Morishima, K., Matsuura, S., Kobayashi, T., Tamai, K., Tanimoto, K. and Komatsu, K. (2002) NBS1 Localizes to γ-H2AX Foci through Interaction with the FHA/BRCT Domain. Current Biol. **12**: 1846–1851.
32. Sakamoto, S., Iijima, K., Mochizuki, D., Nakamura, K., Teshigawara, K., Kobayashi, J., Matsuura, S., Tauchi, H. and Komatsu, K. (2007) Homologous recombination repair is regulated by domains at the N- and C-terminus of NBS1 and is dissociated with ATM functions. Oncogene **26**: 6002–6009.
33. Uchimura, Y., Nakamura, M., Sugasawa, K., Nakao, M. and Saitoh, H. (2004) Overproduction of eukaryotic SUMO-1- and SUMO-2-conjugated proteins in Escherichia coli. Anal. Biochem. **331**: 204–206.
34. Osman, F. and Whitby, M. C. (2007) Exploring the roles of Mus81-Eme1/Mms4 at perturbed replication forks. DNA Repair **6**: 1004–1017.
35. Doe, C. L., Ahn, J. S., Dixon, J. and Whitby, M. C. (2002) Mus81-Eme1 and Rqh1 involvement in processing stalled and collapsed replication forks. J. Biol. Chem. **277**: 32753–32759.
36. Abraham, J., Lemmers, B., Hande, M. P., Moynahan, M. E., Chahwan, C., Ciccia, A., Essers, J., Hanada, K., Chahwan, R., Khaw, A. K., McPherson, P., Shehabeldin, A., Laister, R., Arrowsmith, C., Kanaar, R., West, S. C., Jasin, M. and Hakem, R. (2003) Eme1 is involved in DNA damage processing and maintenance of genomic stability in mammalian cells. EMBO J. **22**: 6137–6147.
37. Dendouga, N., Gao, H., Moechars, D., Janicot, M., Vialard, J. and McGowan, C. H. (2005) Disruption of murine Mus81 increases genomic instability and DNA damage sensitivity but does not promote tumorigenesis. Mol. Cell. Biol. **25**: 7569–7579.
38. Hiyama, T., Katsura, M., Yoshihara, T., Ishida, M., Kinomura, A., Tonda, T., Asahara, T. and Miyagawa, K. (2006) Haploinsufficiency of the Mus81-Eme1 endonuclease activates the intra-S-phase and G2/M checkpoints and promotes rereplication in human cells. Nucleic Acids Res. **34**: 880–892.
39. McPherson, J. P., Lemmers, B., Chahwan, R., Pamidi, A., Migon, E., Matysiak-Zablocki, E., Moynahan, M. E., Essers, J., Hanada, K., Poonepalli, A., Sanchez-Sweatman, O., Khokha, R., Kanaar, R., Jasin, M., Hande, M. P. and Hakem R. (2004) Involvement of mammalian Mus81 in genome integrity and tumor suppression. Science **304**: 1822–1826.
40. Pamidi, A., Cardoso, R., Hakem, A., Matysiak-Zablocki, E., Poonepalli, A., Tamblyn, L., Perez-Ordonez, B., Hande, M. P., Sanchez, O. and Hakem, R. (2007) Functional interplay of p53 and Mus81 in DNA damage responses and cancer. Cancer Res. **67**: 8527–8535.
41. Wang, W. (2007) Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. Nat. Rev. Genet. **8**: 735–748.
42. Sasaki, M. S. and Tonomura, A. (1973) A high susceptibility of Fanconi's anemia to chromosome breakage by DNA cross-linking agents. Cancer Res. **33**: 1829–1836.
43. Takata, M., Sasaki, M. S., Sonoda, E., Fukushima, T., Morrison, C., Albala, J. S., Swagemakers, S. M., Kanaar, R., Thompson, L. H. and Takeda, S. (2000) The Rad51 paralog Rad51B promotes homologous recombinational repair. Mol. Cell. Biol. **20**: 6476–6482.
44. Takata, M., Sasaki, M. S., Tachiiri, S., Fukushima, T., Sonoda, E., Schild, D., Thompson, L. H. and Takeda, S. (2001) Chromosome instability and defective recombinational repair in knockout mutants of the five Rad51 paralogs. Mol. Cell. Biol. **21**: 2858–2866.
45. Venkitaraman, A. R. (2002) Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell **108**: 171–182.
46. Taniguchi, T., Garcia-Higuera, I., Andreassen, P. R., Gregory, R. C., Grompe, M. and D'Andrea, A. D. (2002) S-phase-specific interaction of the Fanconi anemia protein, FANCD2, with BRCA1 and RAD51. Blood **100**: 2414–2420.
47. Garcia-Higuera, I., Taniguchi, T., Ganesan, S., Meyn, M. S., Timmers, C., Hejna, J., Grompe, M. and D'Andrea, A. D. (2001) Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. Mol. Cell **7**: 249–262.
48. Seki, S., Ohzeki, M., Uchida, A., Hirano, S., Matsushita, N., Kitao, H., Oda, T., Yamashita, T., Kashihara, N., Tsubahara, A., Takata, M. and Ishiai, M. (2007) A requirement of FancL and FancD2 monoubiquitination in DNA repair. Genes Cells **12**: 299–310.
49. Takata, M., Kitao, H. and Ishiai, M. (2007) Fanconi anemia: genetic analysis of a human disease using chicken system. Cytogenet. Genome Res. **117**: 346–351.
50. Yamamoto, K., Ishiai, M., Matsushita, N., Arakawa, H., Lamerdin, J. E., Buerstedde, J. M., Tanimoto, M., Harada, M., Thompson, L. H. and Takata, M. (2003) Fanconi anemia FANCG protein in mitigating radiation- and enzyme-induced DNA double-strand breaks by homologous recombination in vertebrate cells. Mol. Cell. Biol. **23**: 5421-5430.
51. Yamamoto, K., Hirano, S., Ishiai, M., Morishima, K., Kitao, H., Namikoshi, K., Kimura, M., Matsushita, N., Arakawa, H., Buerstedde, J. M., Komatsu, K., Thompson, L. H. and Takata, M. (2005) Fanconi anemia protein FANCD2 promotes immunoglobulin gene conversion and DNA repair through a mechanism related to homologous recombination. Mol. Cell. Biol. **25**: 34–43.
52. Arakawa, H. and Buerstedde, J. M. (2004) Immunoglobulin gene conversion: Insights from bursal B cells and the DT40 cell line. Dev. Dyn. **229**: 458–464.
53. Buerstedde, J. M., Reynaud, C. A., Humphries, E. H., Olson, W., Ewert, D. L. and Weill, J. C. (1990) Light chain gene conversion continues at high rate in an ALV-induced cell line. EMBO J. **9**: 921–927.
54. Takata, M., Sasaki, M. S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A. and Takeda, S. (1998) Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. EMBO J. **17**: 5497–5508.
55. Ira, G., Pellicioli, A., Balijja, A., Wang, X., Fiorani, S.,

Carotenuto, W., Liberi, G., Bressan, D., Wan, L., Hollingsworth, N. M., Haber, J. E. and Foiani, M. (2004) DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1. Nature **431**: 1011–1017.

56. Limbo, O., Chahwan, C., Yamada, Y., de Bruin, R. A., Wittenberg, C. and Russell, P. (2007) Ctp1 is a cell-cycle-regulated protein that functions with Mre11 complex to control double-strand break repair by homologous recombination. Mol. Cell **28**: 134–146.
57. Sartori, A. A., Lukas, C., Coates, J., Mistrik, M., Fu, S., Bartek, J., Baer, R., Lukas, J. and Jackson, S. P. (2007) Human CtIP promotes DNA end resection. Nature **450**: 509–514.
58. Fukushima, T., Takata, M., Morrison, C., Araki, R., Fujimori, A., Abe, M., Tatsumi, K., Jasin, M., Dhar, P. K., Sonoda, E., Chiba, T. and Takeda, S. (2001) Genetic analysis of the DNA-dependent protein kinase reveals an inhibitory role of Ku in late S-G2 phase DNA double-strand break repair. J. Biol. Chem. **276**: 44413–44418.
59. Kitao, H., Yamamoto, K., Matsushita, N., Ohzeki, M., Ishiai, M. and Takata, M. (2006) Functional interplay between BRCA2/FANCD1 and FANCC in DNA repair. J. Biol. Chem. **281**: 21312–21320.
60. Takata, M., Yamamoto, K., Matsushita, N., Kitao, H., Hirano, S. and Ishiai, M. (2006) The fanconi anemia pathway promotes homologous recombination repair in DT40 cell line. Subcell Biochem **40**: 295–311.
61. Kannouche, P. L., Wing, J. and Lehmann, A. R. (2004). Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. Mol. Cell **14**: 491–500.
62. Watanabe, K., Tateishi, S., Kawasuji, M., Tsurimoto, T., Inoue, H. and Yamaizumi, M. (2004). Rad18 guides poleta to replication stalling sites through physical interaction and PCNA monoubiquitination. EMBO J. **23**: 3886–3896.
63. Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C. and Chen, Z. J. (2000). Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell **103**: 351–361.
64. Spence, J., Gali, R. R., Dittmar, G., Sherman, F., Karin, M. and Finley, D. (2000). Cell cycle-regulated modification of the ribosome by a variant multiubiquitin chain. Cell **102**: 67–76.
65. Zhao, G. Y., Sonoda, E., Barber, L. J., Oka, H., Murakawa, Y., Yamada, K., Ikura, T., Wang, X., Kobayashi, M., Yamamoto, K., Boulton, S. J. and Takeda, S. (2007). A Critical Role for the Ubiquitin-Conjugating Enzyme Ubc13 in Initiating Homologous Recombination. Mol. Cell **25**: 663–675.
66. Ikura, T., Tashiro, S., Kakino, A., Shima, H., Jacob, N., Amunugama, R., Yoder, K., Izumi, S., Kuraoka, I., Tanaka, K., Kimura, H., Ikura, M., Nishikubo, S., Ito, T., Muto, A., Miyagawa, K., Takeda, S., Fishel, R., Igarashi, K. and Kamiya, K. (2007). DNA damage-dependent acetylation and ubiquitination of H2AX enhances chromatin dynamics. Mol. Cell Biol. **27**: 7028–7040.
67. Murakawa, Y., Sonoda, E., Barber, L. J., Zeng, W., Yokomori, K., Kimura, H., Niimi, A., Lehmann, A., Zhao, G. Y., Hochegger, H., Boulton, S. J. and Takeda, S. (2007). Inhibitors of the proteasome suppress homologous DNA recombination in Mammalian cells. Cancer Res. **67**: 8536–8543.
68. Suzuki, K., Okada, H., Yamauchi, M., Oka, Y., Kodama, S. and Watanabe, M. (2006) Qualitative and quantitative analysis of phosphorylated ATM foci induced by low-dose ionizing radiation. Radiat. Res. **165**: 499–504.
69. Downs, J. A., Nussenzweig, M. C. and Nussenzweig, A. (2007) Chromatin dynamics and the preservation of genetic information. Nature 447: 951–958.
70. Lieber, M. R., Ma, Y., Pannicke, U. and Schwarz, K. (2004) The mechanism of vertebrate nonhomologous DNA end joining and its role in V(D)J recombination. DNA Repair **3**: 817–826.
71. McCabe, N., Turner, N. C., Lord, C. J., Kluzek, K., Bialkowska, A., Swift, S., Giavara, S., O'Connor, M. J., Tutt, A. N., Zdzienicka, M. Z., Smith, G. C. and Ashworth, A. (2006) Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose) polymerase inhibition. *Cancer Res.* **66**: 8109–8115.
72. Tauchi, H., Matsuura, S., Kobayashi, J., Sakamoto, S. and Komatsu, K. (2002) Nijmegen breakage syndrome gene, NBS1, and molecular links to factors for genome stability. *Oncogene* **21**: 8967–8680.
73. Chan, D. W., Chen, B. P., Prithivirajsingh, S., Kurimasa, A., Story, M. D., Qin, J. and Chen, D. J. (2002) Autophosphorylation of the DNA-dependent protein kinase catalytic subunit is required for rejoining of DNA double-strand breaks. *Genes Dev.* **16**: 2333–2338.

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DHMEQ, a novel NF-kappaB inhibitor, suppresses growth and type I collagen accumulation in keloid fibroblasts

Sachio Makino [a], Norisato Mitsutake [b,*], Masahiro Nakashima [c], Vladimir A. Saenko [d], Akira Ohtsuru [e], Kazuo Umezawa [f], Katsumi Tanaka [a], Akiyoshi Hirano [a], Shunichi Yamashita [b,d]

[a] *Division of Plastic and Reconstructive Surgery, Department of Developmental and Reconstructive Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan*
[b] *Department of Molecular Medicine, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan*
[c] *Tissue and Histopathology Section, Division of Scientific Data Registry, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan*
[d] *Department of International Health and Radiation Research, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan*
[e] *Takashi Nagai Memorial International Hibakusha Medical Center, Nagasaki University Hospital, Nagasaki, Japan*
[f] *Department of Applied Chemistry, Faculty of Science and Technology, Keio University, Yokohama, Japan*

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Summary

Background: Keloid is a benign dermal tumor characterized by proliferation of dermal fibroblasts and overproduction of extracellular matrix (ECM). Nuclear factor kappaB (NF-κB) plays an important role in regulation of inflammation, immune response and cell proliferation. Activation of the NF-κB pathway is thought to be closely linked to abnormal cell proliferation and ECM production in keloid fibroblasts.
Objective: This study was set out to investigate the effects of a novel selective NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), on keloid fibroblasts.
Methods: Primary normal and keloid dermal fibroblasts were used for this study. NF-κB activity was assessed by DNA-binding assay and immunohistochemistry. The effect of DHMEQ was evaluated by cell viability, cell growth and type I collagen accumulation.

* Corresponding author. Tel.: +81 95 819 7116; fax: +81 95 819 7117.
E-mail address: mitsu@nagasaki-u.ac.jp (N. Mitsutake).

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Results: Basal NF-κB activity was constitutively elevated in keloid fibroblasts, indicating that this pathway is involved in keloid pathogenesis. DHMEQ markedly reduced cell proliferation and type I collagen accumulation in keloid fibroblasts.
Conclusion: The inhibition of NF-κB by DHMEQ may be an attractive therapeutic approach for keloids.

1. Introduction

Keloids are fibrous overgrowths due to abnormal wound healing process after skin injury. They are characterized by proliferation of dermal fibroblasts and overproduction of extracellular matrix (ECM). Although keloid is a benign dermal tumor, its management is one of the most challenging clinical problems. Keloids do not regress with time and surgical excision alone results in a high rate of recurrence. Various conservative therapies have been attempted, but definite and effective treatment has not yet been established [1,2].

The ubiquitous nuclear factor kappaB (NF-κB) transcription factor regulates expression of a wide spectrum of genes involved in immune and inflammatory response, cellular proliferation and apoptosis [3–6]. Activation of the NF-κB pathway is implicated in a diverse range of diseases including asthma, rheumatoid arthritis, inflammatory bowel disease and human cancer. NFκB-targeted therapy has been attempted in these diseases and appears to be an effective and useful modality [7]. Recently, it has been reported that the NF-κB pathway is also activated in keloid fibroblasts [8].

The NF-κB family is comprised of NF-κB1 (p50/p105), NF-κB2 (p52/p100), p65 (RelA), RelB and c-Rel. Although many dimeric forms of NF-κB have been identified, the most prevalent activated form of NF-κB is the heterodimer consisting of DNA-binding subunit p50 and transactivation subunit p65. In unstimulated cells, NF-κB is sequestered in the cytoplasm in an inactive form by its inhibitor proteins called inhibitors of κB (IκBs). A variety of stimuli including proinflammatory cytokines, mitogens, viral infection, ultraviolet radiation and free radicals activate a large complex termed IκB kinase (IKK), which phosphorylates IκB protein, resulting in its ubiquitination and proteosomal degradation. Then, freed NF-κB translocates to the nucleus and activates transcription of a vast number of target genes. Some of the proinflammatory cytokines that might have a role in keloid pathogenesis such as interleukin 1 (IL-1) and tumor necrosis factor α (TNF-α) are stimulated by NF-κB. These cytokines can directly activate the NF-κB pathway, thus establishing a positive autoregulatory loop. Hence, the blockade of the NF-κB pathway could be an attractive strategy for the treatment of keloids.

A novel NF-κB inhibitor dehydroxymethylepoxyquinomicin (DHMEQ), a derivative of the antibiotic epoxyquinomicin C [9], has been found to specifically inhibit TNF-α-induced nuclear translocation of NF-κB [10]. Treatment with DHMEQ showed an anti-inflammatory effect on rheumatoid arthritis and renal inflammation in animals. DHMEQ also demonstrated potent anti-cancer activity in various *in vitro* and *in vivo* models such as prostate carcinoma, breast carcinoma, thyroid carcinoma and hematologic malignancies without any apparent toxic side effects [11,12].

In the present study, we report that DHMEQ is effective in blocking growth and type I collagen accumulation in keloid fibroblasts, supporting the concept that inhibitors of the NF-κB pathway may be useful therapeutic agents for keloids.

2. Materials and methods

2.1. Reagents

Stock solutions of racemic DHMEQ (20 mg/ml) were prepared in dimethylsulfoxide (DMSO) and stored at −20 °C until use. Antibodies were obtained from the following sources: anti-p65 polyclonal and β-actin monoclonal from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-collagen type I polyclonal from Rockland Immunochemicals (Gilbertsville, PA, USA); anti-Hsp47 monoclonal from Stressgen Assay Designs (Ann Arbor, MI, USA); anti-Poly ADP ribose polymerase (PARP) polyclonal and secondary horseradish peroxidase (HRP)-conjugated anti-rabbit/mouse IgG from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell culture

Keloid tissue samples were obtained from four different Japanese patients at the time of surgery. Four normal skin tissue samples were obtained from four different Japanese volunteers. No comorbid systemic disorders such as diabetes and cancers, nor taking drugs known to influence the transcriptional

response of cells, were identified. All experiments were performed after obtaining hospital ethical committee approval. Informed consent was obtained from each individual. The profile of each sample is summarized in Table 1. All keloid tissue specimens were pathologically examined to confirm the diagnosis.

Primary cultures of dermal fibroblasts were established as previously described [13]. Explants were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% (w/v) penicillin/streptomycin in 5% CO_2 humidified atmosphere at 37 °C. Fibroblasts at passages of 3–8 were used in this study. We did not observe any alteration in cell shape, growth rate and sensitivity to DHMEQ until passage 8.

2.3. DNA-binding assay

Nuclear extracts were prepared as described previously [14]. The multiwell colorimetric assay for phosphorylated p65 protein was performed using the Trans-AM NF-κB p65 Transcription Factor Assay Kit (Active Motif North America, Carlsbad, CA, USA). Briefly, equal amount of nuclear extracts were incubated in 96-well plates coated with immobilized oligonucleotide containing a NF-κB consensus binding site. NF-κB binding to target oligonucleotides was detected by incubation with primary antibodies against the p65 subunit and HRP-conjugated secondary antibody. For quantification of activity, optical densities were measured at 450 nm with a microplate reader ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan).

2.4. Immunohistochemistry

Immunohistochemical detection of NF-κB p65 subunit was done using immunoperoxidase technique. Briefly, tissue sections from paraffin-embedded tissue were deparaffinized in xylene and dehydrated in alcohol. Slides were then subjected to antigen retrieval by four times of 5-min microwave heating in 10 mM sodium citrate (pH 6.0) and cooled at room temperature. Endogenous peroxidase was blocked by incubation for 10 min with 3% hydrogen peroxidase in deionized water. Nonspecific binding was blocked with 1% BSA for 10 min. Sections were then incubated overnight at 4 °C with anti-p65 polyclonal antibody. Biotinylated secondary antibody was applied, and sections were incubated with avidin–biotin peroxidase complex, developed with 3,3′-diaminobenzidine substrate and counterstained with hematoxylin. Positive cells were counted in six high power fields (magnification ×400) by two independent investigators in a blinded fashion.

2.5. Cell viability assay

Cultures were established in 96-well flat-bottomed microtiter plates (Nalge Nunc International, Tokyo, Japan). Cells were counted, plated at 5×10^3 cells/well (100 μl) and incubated for 24 h before treatment. Solutions containing DHMEQ were added to each well in 10 μl of medium at various concentrations, with six wells used for each concentration. In control wells, solution of DMSO was added. Cells were incubated at 37 °C for 24 h, and then water-soluble tetrazolium salt (WST)-based assay was done as follows: medium was changed (100 μl/well), and 10 μl of Cell Counting Kit-8 solution (Dojindo, Kumamoto, Japan) were added to each well and incubated for 2 h at 37 °C. Optical densities were measured at 450 nm in a microplate reader ImmunoMini NJ-2300.

2.6. Cell proliferation assay

Cell suspensions (100 μl, 1×10^3 cells/well) were added to each well of 96-well flat-bottomed microtiter plates and incubated for 24 h before treatment. Solutions of DHMEQ were added to each wells at the

Table 1 The profile of each sample for primary culture

	Sex	Age (years)	Biopsy site	Duration of the lesion (years)
Normal				
NF1	Female	20	Back	–
NF2	Female	77	Thigh	–
NF3	Male	22	Thigh	–
NF4	Male	64	Back	–
Keloid				
KF1	Female	29	Ear	3
KF2	Male	38	Chest	6
KF3	Male	64	Chest	8
KF4	Female	28	Shoulder	2

concentration of 5 or 10 μg/ml. After 24, 48, 96 h exposure to the drug, WST-based assay was done as described above.

2.7. Western blotting

Cells were washed twice with ice-cold PBS, collected in 1 ml PBS and centrifuged for 3 min at 3000 rpm. For total cell extracts, each pellet was resuspended in 200 μl of a buffer containing 20 mM HEPES (pH 7.5), 0.35 M NaCl, 20% glycerol, 1% NP40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA and protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). After incubated for 15 min on ice, lysates were centrifuged for 15 min at 15,000 rpm at 4 °C, and the supernatants were collected and stored at −80 °C. To obtain the secreted ECM proteins, culture media was collected and concentrated using a Speed Vac CVE 200D (EYELA, Tokyo, Japan). Protein concentrations were determined with a Bichinonic Acid Assay Kit (Sigma, St Louis, MO, USA). Equal amount of proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA) by semidry blotting. After incubation with appropriate primary antibody, the antigen–antibody complexes were visualized using HRP-conjugated secondary antibody and enhanced chemiluminescence reagents (Amersham, Piscataway, NJ, USA). For quantitation of band intensities, image analysis was done with Gel-Pro Analyzer software (Media Cybernetics, Bethesda, MD, USA).

2.8. Statistical analysis

All data were expressed as the mean ± S.D. Differences between groups were examined for statistical significance with the Mann–Whitney *U* test and/or one-way ANOVA followed by Fisher's PLSD where appropriate. A *P* value not exceeding 0.05 was considered statistically significant.

3. Results

3.1. NF-κB is activated in keloid fibroblasts

We first examined basal DNA-binding activity of NF-κB in normal and keloid fibroblasts. For this purpose, primary fibroblasts derived from four different patients and healthy volunteers were established. Consistent with the previous report [8], there was elevated basal level of NF-κB activity in keloid fibroblasts as compared to normal fibroblasts (Fig. 1).

To further confirm activation of the NF-κB pathway in keloids, we performed immunohistochemistry using keloid tissue specimens. As shown in Fig. 2, intense immunoreactivity for p65 in both cytoplasm and nucleus was observed in keloid tissue (Fig. 2B and D). By contrast, normal skin tissue specimen showed very weak immunoreactivity (Fig. 2A and C). The number of positive cells in keloid tissue was significantly higher than that in normal tissue (Fig. 2E). Note that epidermis of the keloid tissue section also showed diffuse and intense immunostaining for p65, suggesting that NF-κB is also activated in keloid keratinocytes. Thus, activation of NF-κB in keloids was confirmed both *in vitro* and *in vivo*.

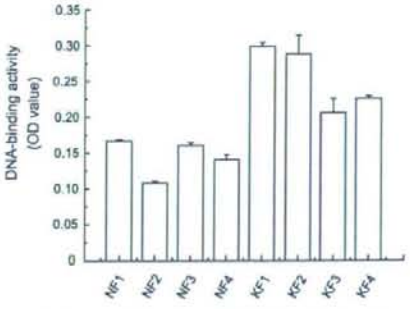

Fig. 1 Basal NF-κB DNA-binding activity of normal and keloid fibroblasts. p65 DNA-binding activity was measured using nuclear extracts isolated from primary normal fibroblasts (NF1-4) and keloid fibroblasts (KF1-4) as described in Section 2. Results are presented as the mean ± S.D. of the data collected in triplicate.

3.2. DHMEQ suppresses NF-κB in keloid fibroblasts

To examine the pharmacological effect of DHMEQ on primary normal and keloid fibroblasts, DNA-binding assay was performed. The addition of DHMEQ was found to suppress basal DNA binding of the p65 protein in both normal and keloid fibroblasts; however, keloid fibroblasts were more sensitive to DHMEQ (Fig. 3). Although the mean basal NF-κB activity was approximately 1.7-fold higher in keloid fibroblasts compared to normal fibroblasts ($P < 0.05$), it decreased to the same level as in normal fibroblasts after treated with 5 μg/ml of DHMEQ. This result suggests that DHMEQ successfully suppressed NF-κB activity in keloid fibroblasts.

3.3. Cytotoxic effect of DHMEQ

To determine the cytotoxic effect of DHMEQ, normal and keloid fibroblasts were treated with various concentrations of DHMEQ, and cell viabi-

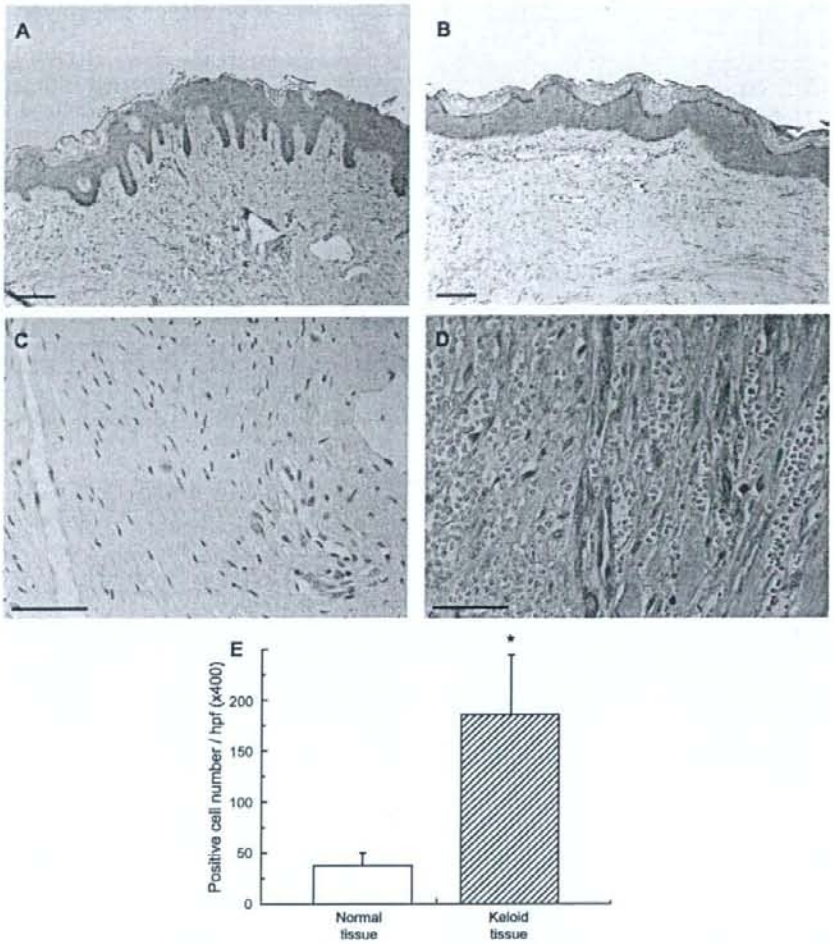

Fig. 2 Representative immunostaining for p65 in normal and keloid tissues. (A and C) Normal skin specimen. (B and D) Keloid tissue specimen. (A and B) Original magnification: ×100; bars: 100 μm. (C and D) Original magnification: ×400; bars: 50 μm. Similar results were obtained in all cases. (E) The number of positive cells in each high power field (hpf) was counted under microscope at magnification of ×400. Data are presented as the mean ± S.D. of six different fields. *$P < 0.05$ vs. normal tissue.

lity was estimated by WST assay. The response varied between the cells obtained from different individuals, but viability of keloid fibroblasts was decreased stronger than that of normal fibroblasts at same concentration of DHMEQ (Fig. 4). Average ED_{50} was calculated to be 27.3 ± 4.0 μg/ml for normal fibroblasts and 20.1 ± 2.9 μg/ml for keloid fibroblasts ($P < 0.05$). To explore whether the cytotoxic effect of DHMEQ was due to apoptosis, we performed Western blotting for PARP. Cleavage of PARP along with DNA laddering and membrane inversion are known to be the most characteristic events in apoptosis. As shown in Fig. 4B, apparent PARP cleavage was detected from the concentration of 25 μg/ml in both normal and keloid fibroblasts. DHMEQ did not induce any cytotoxic effect at lower than 15 μg/ml in normal fibroblasts. Thus, we used DHMEQ at the concentration of 5 and 10 μg/ml for further experiments.

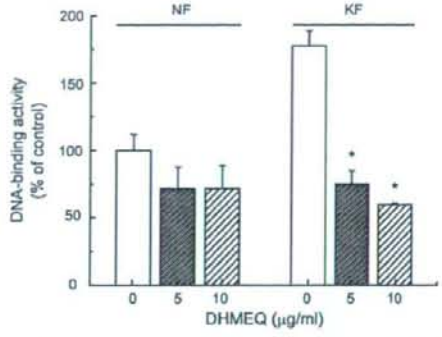

Fig. 3 Inhibition of NF-κB DNA-binding activity by DHMEQ. Primary normal fibroblasts (NF) or keloid fibroblasts (KF) were treated with DHMEQ for 1 h. Nuclear extracts were used for binding assays as described in Section 2. Results are shown as percentage relative to the normal skin control. Data are presented as the mean ± S.D. of four different lines (NF1-4 and KF1-4). *$P < 0.05$ vs. control.

3.4. Effect of DHMEQ on cell growth

We next examined the effect of DHMEQ on cell proliferation. Cells were treated with DHMEQ at the concentration of 5 or 10 μg/ml and cultured for up to 4 days. Although the response to DHMEQ varied between the cells, most of normal fibroblasts were affected only slightly (Fig. 5A), and the DHMEQ treatment had no significant influence on average cell proliferation of normal fibroblasts (Fig. 5B). By contrast, the growth of keloid fibroblasts was significantly inhibited compared to the normal fibroblasts (Fig. 5A and B) in a dose dependent manner. It has been reported that DHMEQ causes only a transient NF-κB inhibition [15], but DHMEQ inhibited the growth of keloids during the observed period.

3.5. Effect of DHMEQ on type I collagen accumulation

To study the effect of DHMEQ on ECM, we performed Western blotting to assess both secreted and cellu-

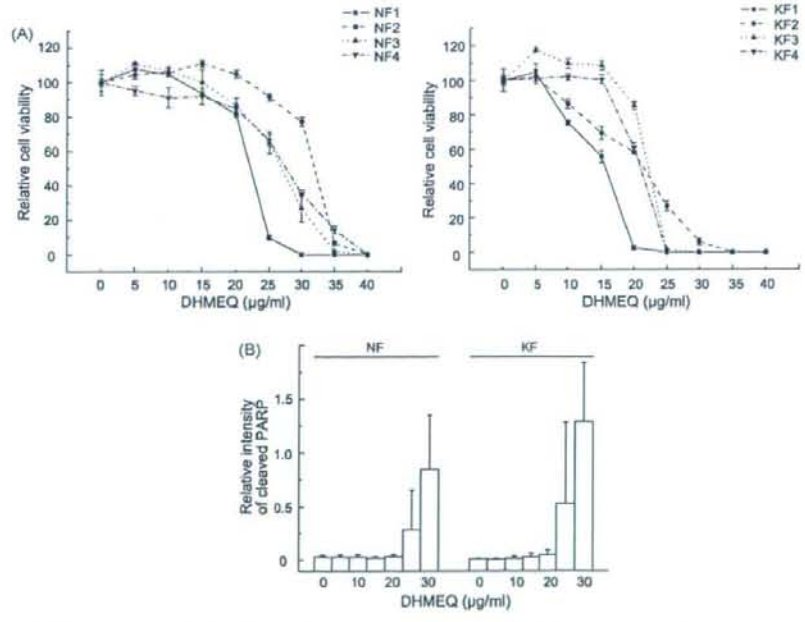

Fig. 4 Induction of cell death by DHMEQ. (A) Viability of normal and keloid fibroblasts after treatment with various concentrations of DHMEQ for 24 h was estimated by WST-based assay. Each point indicates mean ± S.D. of the data collected from six wells. (B) Cells were exposed to various concentrations of DHMEQ for 24 h, and Western blotting for PARP was performed using total cell lysates. Densitometric analysis was done and the level of PARP cleavage was expressed as a ratio of cleaved band to uncleaved band of untreated cells. Data are presented as mean ± S.D. of four different lines (NF1-4 and KF1-4). Similar results were obtained in three independent experiments.

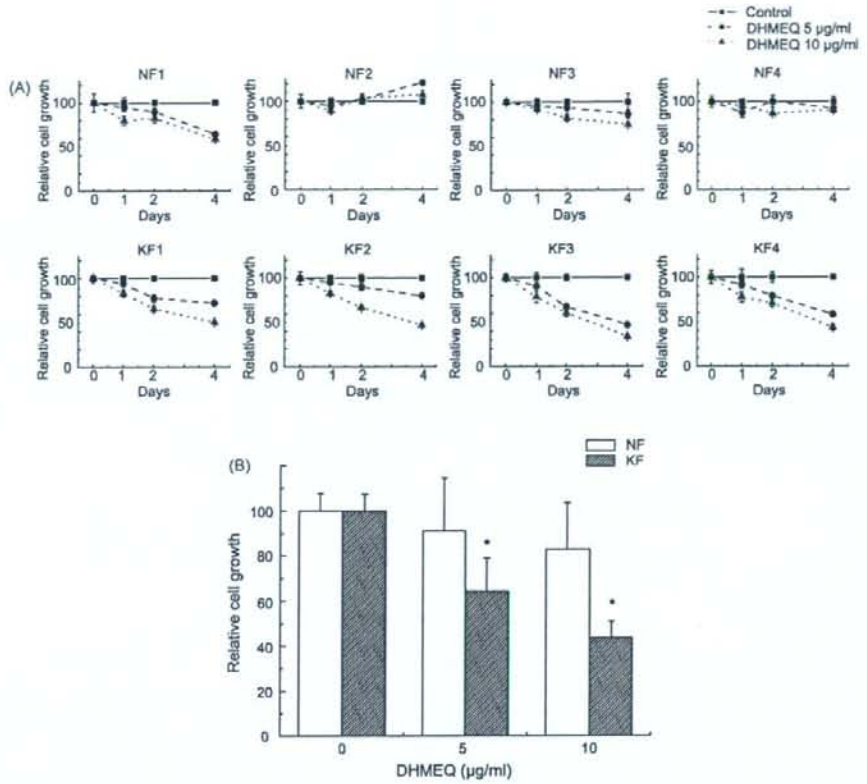

Fig. 5 Effect of DHMEQ on cell growth. (A) Cells were treated with 5 or 10 μg/ml of DHMEQ and cultured for up to 4 days. Each point indicates the mean ± S.D. of the data collected from six wells. (B) Relative cell number at day 4. Data are presented as mean ± S.D. of four different lines (NF1-4 and KF1-4). $^*P < 0.05$ vs. normal fibroblasts. Similar results were obtained in three independent experiments.

lar type I collagen. The culture media or whole cell lysates were used for this purpose. After 24 h treatment with DHMEQ, expression of both secreted and cellular type I collagen were reduced in keloid fibroblasts in a dose dependent manner, while normal fibroblasts showed no decrease (Fig. 6).

HSP47 is a collagen-specific molecular chaperone that interacts with procollagen during the process of folding, assembly and transport from the endoplasmic reticulum [16]. The expression of HSP47 is closely correlated with that of collagen in fibrotic disorders including keloids. However, the exact mechanism of how HSP47 is regulated remains unclear. Therefore, we performed Western blotting to investigate whether DHMEQ affects HSP47 expression. Consistent with a previous report [17], very strong immunoreactive bands at 47 kDa were detected in all keloid samples compared to normal samples. However, DHMEQ treatment did not show any effect on HSP47 expression (Fig. 6), indicating that the regulation of HSP47 is independent of NF-κB signaling pathway.

4. Discussion

In the present study, we demonstrated the constitutive elevation of the NF-κB activity in keloid fibroblasts in both DNA-binding assay (Fig. 1) and immunohistochemical analysis (Fig. 2), suggesting that this signaling pathway may play a role in keloid pathogenesis. Since NF-κB family protein is found in virtually all cell types, normal fibroblasts are also affected by DHMEQ. However, cell viability assay showed no significant cell number decrease at lower than 15 μg/ml of DHMEQ in normal fibroblasts

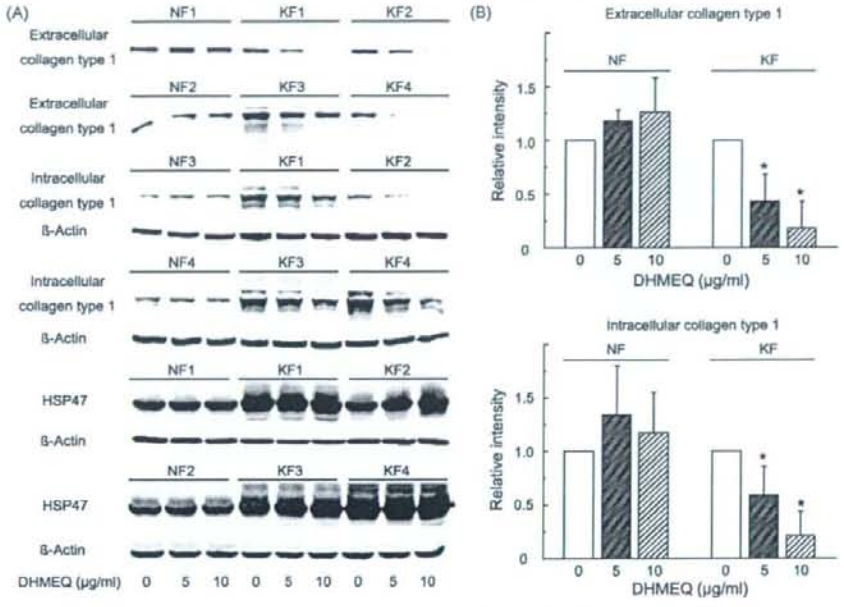

Fig. 6 Effect of DHMEQ on type I collagen accumulation. (A) Representative blot images. Indicated cells were treated with 5 or 10 μg/ml of DHMEQ for 24 h. Cultured media (extracellular type I collagen) or total cell lysates (intracellular type I collagen and HSP47) were analyzed by Western blotting. β-actin was used as a loading control. (B) Results of densitometric analysis of detected bands. Results are expressed as relative levels compared to those of untreated cells. Data are presented as mean ± S.D. of four different lines (NF1-4 and KF1-4). $^*P < 0.05$ vs. untreated cells. Similar results were obtained in three independent experiments.

(Fig. 4A), and also cell proliferation assay showed that only keloid fibroblasts displayed significant growth reduction at low doses of DHMEQ (Fig. 5). Presumably, this could be due to low basal NF-κB activity in normal fibroblasts since low concentration of DHMEQ hardly affected NF-κB activity in normal fibroblasts but strongly reduced in keloid fibroblasts (Fig. 3). These results suggest that survival and growth of keloid fibroblasts are largely dependent on high basal activity of NF-κB signaling.

Two mechanisms can be considered for how DHMEQ suppresses proliferation of keloid fibroblasts. One is through down-regulation of genes involved in cell cycle progression. Cyclins D1 and D2 are downstream targets of NF-κB pathway and directly regulate G1-S transition [18]. Thus, the down-regulation of those cyclins by DHMEQ may lead to growth arrest in keloid fibroblasts. The other is through suppression of inflammatory cytokine IL-6. IL-6 is also a downstream target of NF-κB pathway and is involved in keloid pathogenesis. Because IL-6 peptide induces proliferation of fibroblasts [19], inhibition of this cytokine by DHMEQ may also lead to decreased proliferation of keloid fibroblasts.

The effect of NF-κB activation on type I collagen, a principal component of excessive matrix in keloids, seems to be complicated. Type I collagen is a heterotrimeric protein composed of two α1 chains and one α2 chain encoded by the *COL1A1* and *COL1A2* genes, respectively. Several reports have proposed that NF-κB regulates type I collagen negatively. NF-κB binds to the site in *COL1A1* and *COL1A2* promoters, leading to inhibition of transcriptional activity [20,21]. Furthermore, activated NF-κB suppresses the TGF-β/Smad pathway which promotes fibrosis by inducing inhibitory Smad7 protein [22,23]. On the contrary, it has been found that IL-4-mediated activation of the *COL1A2* promoter needs cooperation with NF-κB in human embryonic lung fibroblasts [24], and *COL1A2* promoter activation by TGF-β also requires NF-κB binding to its consensus site [25]. These facts imply that NF-κB has dual role in type I collagen regulation, presumably depending on cell/tissue circumstances. However, the main role of activated NF-κB in keloid fibroblasts seems to be the positive regulation of type I collagen accumulation, thus its inhibition by DHMEQ resulted in decreased accumulation of type I

collagen (Fig. 6). Our result suggests another contribution of NF-κB pathway to keloid pathogenesis.

A variety of anti-inflammatory agents are known to affect NF-κB activity. For example, steroids, the most widely used drugs in keloid treatment, increase the expression of IκBα, resulting in the cytoplasmic retention of NF-κB [26,27]. In parallel, direct protein–protein interaction between the activated glucocorticoid receptor and NF-κB seems to suppress the activation of its pathway [28]. Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and sodium salicylate also suppress NF-κB pathway by inhibiting ATP binding to IKKβ [29]. Recently, Zhu et al. have reported that aspirin successfully attenuated the cell proliferation and enhanced TNF-α-mediated apoptosis in keloid fibroblasts [30]. However, it is evident that steroids and NSAIDs are not specific for IKK nor NF-κB. To the best of our knowledge, DHMEQ is the most specific inhibitor that exclusively suppresses NF-κB translocation from the cytoplasm to the nucleus. Together, these facts and our results suggest that NF-κB-targeted therapy by DHMEQ may be a very attractive approach to the management of keloids.

One concern about inhibiting the NF-κB pathway in skin tumors including keloids is that NF-κB seems to have distinct role in epidermal keratinocytes. It has been reported that NF-κB activation is required for normal differentiation of keratinocytes [31], and inhibition of the NF-κB pathway in murine epidermis resulted in hyperproliferation of keratinocytes and spontaneous development of squamous cell carcinomas [32–34]. However, in keloids, not only fibroblasts but also keratinocytes seem to be abnormal and contribute to keloid pathogenesis [35,36]. Consistent with this, our results of immunohistochemical analysis suggested that NF-κB is activated in overlying epidermis of keloid lesion as well (Fig. 2B). Thus, topical treatment with NF-κB inhibitor might be ideally suited to keloid, although further assessment of NF-κB function in other type of cells, e.g. keratinocytes would be necessary.

In conclusion, our results provide a new insight into keloid pathogenesis and therapeutic strategy. DHMEQ effectively suppressed the growth of keloid fibroblasts and type I collagen accumulation through the inhibition of NF-κB activity, suggesting that the inhibition of NF-κB by DHMEQ may be an attractive treatment modality for keloids.

Acknowledgement

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References

[1] Al-Attar A, Mess S, Thomassen JM, Kauffman CL, Davison SP. Keloid pathogenesis and treatment. Plast Reconstr Surg 2006;117:286–300.
[2] Atiyeh BS, Costagliola M, Hayek SN. Keloid or hypertrophic scar: the controversy: review of the literature. Ann Plast Surg 2005;54:676–80.
[3] Ghosh S, May MJ, Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol 1998;16:225–60.
[4] Luo JL, Kamata H, Karin M. IKK/NF-kappaB signaling: balancing life and death—a new approach to cancer therapy. J Clin Invest 2005;115:2625–32.
[5] Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 1999;18:6853–66.
[6] Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. J Clin Invest 2001;107:135–42.
[7] Baldwin Jr AS. Series introduction: the transcription factor NF-kappaB and human disease. J Clin Invest 2001;107:3–6.
[8] Messadi DV, Doung HS, Zhang Q, et al. Activation of NFkappaB signal pathways in keloid fibroblasts. Arch Dermatol Res 2004;296:125–33.
[9] Matsumoto N, Ariga A, To-e S, et al. Synthesis of NF-kappaB activation inhibitors derived from epoxyquinomicin C. Bioorg Med Chem Lett 2000;10:865–9.
[10] Ariga A, Namekawa J, Matsumoto N, Inoue J, Umezawa K. Inhibition of tumor necrosis factor-alpha-induced nuclear translocation and activation of NF-kappa B by dehydroxymethylepoxyquinomicin. J Biol Chem 2002;277:24625–30.
[11] Umezawa K. Inhibition of tumor growth by NF-kappaB inhibitors. Cancer Sci 2006;97:990–5.
[12] Palona I, Namba H, Mitsutake N, et al. BRAFV600E promotes invasiveness of thyroid cancer cells through nuclear factor kappaB activation. Endocrinology 2006;147:5699–707.
[13] Arakawa M, Hatamochi A, Takeda K, Ueki H. Increased collagen synthesis accompanying elevated m-RNA levels in cultured Werner's syndrome fibroblasts. J Invest Dermatol 1990;94:187–90.
[14] Li N, Karin M. Signaling pathways leading to nuclear factor-kappa B activation. Methods Enzymol 2000;319:273–9.
[15] Kikuchi E, Horiguchi Y, Nakashima J, et al. Suppression of hormone-refractory prostate cancer by a novel nuclear factor kappaB inhibitor in nude mice. Cancer Res 2003;63:107–10.
[16] Nagata K. Hsp47: a collagen-specific molecular chaperone. Trends Biochem Sci 1996;21:22–6.
[17] Naitoh M, Hosokawa N, Kubota H, et al. Upregulation of HSP47 and collagen type III in the dermal fibrotic disease, keloid. Biochem Biophys Res Commun 2001;280:1316–22.
[18] Watanabe M, Dewan MZ, Okamura T, et al. A novel NF-kappaB inhibitor DHMEQ selectively targets constitutive NF-kappaB activity and induces apoptosis of multiple myeloma cells in vitro and in vivo. Int J Cancer 2005;114:32–8.
[19] Ghazizadeh M, Tosa M, Shimizu H, Hyakusoku H, Kawanami O. Functional implications of the IL-6 signaling pathway in keloid pathogenesis. J Invest Dermatol 2007;127:98–105.
[20] Kouba DJ, Chung KY, Nishiyama T, et al. Nuclear factor-kappa B mediates TNF-alpha inhibitory effect on alpha 2(I) collagen (COL1A2) gene transcription in human dermal fibroblasts. J Immunol 1999;162:4226–34.
[21] Rippe RA, Schrum LW, Stefanovic B, Solis-Herruzo JA, Brenner DA. NF-kappaB inhibits expression of the alpha1(I) collagen gene. DNA Cell Biol 1999;18:751–61.

[22] Bitzer M, von Gersdorff G, Liang D, et al. A mechanism of suppression of TGF-beta/SMAD signaling by NF-kappa B/RelA. Genes Dev 2000;14:187–97.
[23] Ishida Y, Kondo T, Kimura A, Matsushima K, Mukaida N. Absence of IL-1 receptor antagonist impaired wound healing along with aberrant NF-kappaB activation and a reciprocal suppression of TGF-beta signal pathway. J Immunol 2006;176:5598–606.
[24] Buttner C, Skupin A, Rieber EP. Transcriptional activation of the type I collagen genes COL1A1 and COL1A2 in fibroblasts by interleukin-4: analysis of the functional collagen promoter sequences. J Cell Physiol 2004;198:248–58.
[25] Novitskiy G, Potter JJ, Rennie-Tankersley L, Mezey E. Identification of a novel NF-kappaB-binding site with regulation of the murine alpha2(I) collagen promoter. J Biol Chem 2004;279:15639–44.
[26] Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. Science 1995;270:286–90.
[27] Scheinman RI, Cogswell PC, Lofquist AK, Baldwin Jr AS. Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. Science 1995;270:283–6.
[28] De Bosscher K, Schmitz ML, Vanden Berghe W, Plaisance S, Fiers W, Haegeman G. Glucocorticoid-mediated repression of nuclear factor-kappaB-dependent transcription involves direct interference with transactivation. Proc Natl Acad Sci USA 1997;94:13504–9.
[29] Yin MJ, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. Nature 1998;396:77–80.
[30] Zhu G, Cai J, Zhang J, Zhao Y, Xu B. Abnormal nuclear factor (NF)-kappaB signal pathway and aspirin inhibits tumor necrosis factor alpha-induced NF-kappaB activation in keloid fibroblasts. Dermatol Surg 2007;33:697–708.
[31] Seitz CS, Freiberg RA, Hinata K, Khavari PA. NF-kappaB determines localization and features of cell death in epidermis. J Clin Invest 2000;105:253–60.
[32] Dajee M, Lazarov M, Zhang JY, et al. NF-kappaB blockade and oncogenic Ras trigger invasive human epidermal neoplasia. Nature 2003;421:639–43.
[33] Seitz CS, Lin Q, Deng H, Khavari PA. Alterations in NF-kappaB function in transgenic epithelial tissue demonstrate a growth inhibitory role for NF-kappaB. Proc Natl Acad Sci USA 1998;95:2307–12.
[34] van Hogerlinden M, Rozell BL, Ahrlund-Richter L, Toftgard R. Squamous cell carcinomas and increased apoptosis in skin with inhibited Rel/nuclear factor-kappaB signaling. Cancer Res 1999;59:3299–303.
[35] Lim IJ, Phan TT, Song C, Tan WT, Longaker MT. Investigation of the influence of keloid-derived keratinocytes on fibroblast growth and proliferation in vitro. Plast Reconstr Surg 2001;107:797–808.
[36] Lim IJ, Phan TT, Bay BH, et al. Fibroblasts cocultured with keloid keratinocytes: normal fibroblasts secrete collagen in a keloid like manner. Am J Physiol Cell Physiol 2002;283:C212–22.

Effect of Folic Acid and B Vitamins on Cardiovascular Disease in Women

To the Editor: Despite the negative outcomes of the Women's Antioxidant and Folic Acid Cardiovascular Study (WAFACS) by Dr Albert and colleagues[1] and the VISP, HOPE-2, NORVIT, CHAOS-2, and WENBIT trials,[2] we believe that the homocysteine hypothesis is by no means dead. Although pharmacological intervention with B vitamin supplementation does not seem to provide beneficial cardiovascular outcomes, nutritional intervention with fortification of cereal grains with folic acid may have a significant long-term effect on primary cardiovascular prevention.

Hyperhomocysteinemia does not appear to be as important as other cardiovascular risk factors, such as smoking, dyslipidemia, diabetes mellitus, and hypertension, associated with only a small share of the atherosclerotic process.[3] Hence, administration of homocysteine-lowering B vitamin supplements to modify this minor risk factor may require a long period to demonstrate clinical benefits—probably longer than 7.3 years, which was the follow-up duration of the WAFACS trial.[1] Adding 1 more pill to a treatment regimen for such a long period for such a delayed result may not be worthwhile.

On the other hand, fortification of grain products with folic acid exerts a continuous, anti-atherogenic effect that, although modest, would be expected to cumulate over years, possibly leading to a significant long-term reduction in cardiovascular events. Therefore, the ultimate challenge for the homocysteine hypothesis will not necessarily be the ongoing large randomized trials or the preplanned meta-analysis of the B-Vitamin Treatment Trialist's Collaboration.[4] The verdict will be reached with the assessment of the long-term effect of folic acid fortification on cardiovascular risk. Such effects may be observable soon, since fortification in the United States and Canada is already 10 years old.[5]

George Ntaios, MD
ntaiosgeorge@yahoo.gr
Christos Savopoulos, MD
Dimitrios Karamitsos, MD
First Propeudeutic Department of Internal Medicine
AHEPA Hospital
Thessaloniki, Greece

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1. Albert CM, Cook NR, Gaziano JM, et al. Effect of folic acid and B vitamins on risk of cardiovascular events and total mortality among women at high risk for cardiovascular disease: a randomized trial. *JAMA*. 2008;299(17):2027-2036.
2. Lonn E. Homocysteine-lowering B vitamin therapy in cardiovascular prevention: wrong again? *JAMA*. 2008;299(17):2086-2087.
3. Cesari M, Rossi GP, Sticchi D, et al. Is homocysteine important as risk factor for coronary heart disease? *Nutr Metab Cardiovasc Dis*. 2005;15(2):140-147.
4. B-Vitamin Treatment Trialist's Collaboration. Homocysteine-lowering trials for prevention of cardiovascular events. *Am Heart J*. 2006;151(2):282-287.
5. Ntaios GC, Savopoulos CG, Chatzinikolaou AC, et al. Vitamins and stroke: the homocysteine hypothesis still in doubt. *Neurologist*. 2008;14(1):2-4.

To the Editor: Dr Albert and colleagues[1] found that a combination of oral folic acid, vitamin B_6, and vitamin B_{12} did not reduce total cardiovascular events among high-risk women after 7.3 years of treatment and follow-up despite significant homocysteine lowering. However, almost 70% of their cohort had folate concentrations greater than 7 ng/mL before supplementation (to convert folate to nmol/L, multiply by 2.266), suggesting that their prospective study was not performed in a folate-deficient population. In addition, the study population consisted of health professionals, a group at a relatively low risk of folate deficiency.

We have shown that Kazakhstan, located in Central Asia, might be a folate-deficient area.[2] The traditional food of the Kazakh people consists mainly of meat; intake of vegetables tends to be low, especially in rural areas, owing to inadequate distribution. Screening folate concentrations in 50 healthy Kazakh adults were low in 82% of participants, whereas no one in an age-matched Japanese population showed folate deficiency.[2] This suggests that the magnitude of folic acid supplementation may differ in each ethnic group according to its lifestyle and dietary habits. A study conducted in China showed that even within the same country, frequency of folate supplementation differs, which might account for the varying frequency of neural tube defects.[3]

There is general consensus that folic acid supplementation during the periconception period substantially reduces the risk of neural tube defects.[4] Although studies such as that by Albert et al have shown that a combination of folic acid, vitamin B_6, and vitamin B_{12} did not reduce the combined end point of cardiovascular events among high-risk women older than 40 years, we believe that in areas such as Kazakhstan food fortification should definitely be implemented to reduce the possible incidence of adverse health outcomes in the general population. This relates not only to cardiovascular disease but also to congenital anomalies such as neural tube defects. The risk and benefit of fortification should be assessed in each geographic area and in

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each ethnic group on the basis of current nutritional status, eating habits, food distribution, and genetic background.

Ainur Akilzhanova, MD, PhD
Department of Molecular Pathology
Noboru Takamura, MD, PhD
takamura@nagasaki-u.ac.jp
Department of Radiation Epidemiology
Shunichi Yamashita, MD, PhD
Department of Molecular Medicine
Nagasaki University Graduate School of Biomedical Sciences
Nagasaki, Japan

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1. Albert CM, Cook NR, Gaziano JM, et al. Effect of folic acid and B vitamins on risk of cardiovascular events and total mortality among women at high risk for cardiovascular disease: a randomized trial. *JAMA*. 2008;299(17):2027-2036.
2. Akilzhanova A, Takamura N, Zhaojia Y, Aoyagi K, Karazhanova L, Yamashita S. Kazakhstan: a folate deficient area? *Eur J Clin Nutr*. 2006;60(9):1141-1143.
3. Ren A, Zhang L, Hao L, Li Z, Tian Y, Li Z. Comparison of blood folate levels among pregnant Chinese women in areas with high and low prevalence of neural tube defects. *Public Health Nutr*. 2007;10(8):762-768.
4. Tamura T, Piccicano MF. Folate and human reproduction. *Am J Clin Nutr*. 2006; 83(5):993-1016.

To the Editor: Dr Albert and colleagues[1] studied the effect of folic acid and B vitamins on cardiovascular disease. In their study, more than one-fifth of the participants had diabetes mellitus and therefore may have been using oral hypoglycemic agents.

The authors did not describe the use of any diabetes medications in the article. Rosiglitazone, a widely used antidiabetic agent introduced in 1999, could have been prescribed; it is associated with higher risk of myocardial ischemic events.[2] Failure to consider such a possibility could have led to biased measure of the incidence of cardiovascular disease in the study population.

Weekitt Kittisupamongkol, MD
weekitti@gmail.com
Hua Chiew Hospital
Bangkok, Thailand

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1. Albert CM, Cook NR, Gaziano JM, et al. Effect of folic acid and B vitamins on risk of cardiovascular events and total mortality among women at high risk for cardiovascular disease: a randomized trial. *JAMA*. 2008;299(17):2027-2036.
2. Nissen SE, Wolski K. Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes [published correction in *N Engl J Med*. 2007;357(1):100]. *N Engl J Med*. 2007;356(24):2457-2471.

In Reply: We agree with Dr Ntaios and colleagues that null results from published randomized trials of folic acid and B vitamins do not rule out the possibility that long-term dietary folic acid fortification may have a beneficial effect on cardiovascular risk. However, dietary fortification has been broadly implemented in a nonrandomized fashion. It will therefore be difficult to disentangle the specific effects of fortification from other changes and improvements in risk factor profiles and preventive therapies that may also have taken place in these populations over the same time period and that may influence cardiovascular risk.

Also, as Dr Akilzhanova and colleagues point out, the results in our fortified study population of health professionals who were at low risk for folate deficiency may not apply to populations with a greater prevalence of folate deficiency. We agree that dietary fortification with folic acid should be pursued in such populations to reduce possible adverse health outcomes associated with deficiencies, especially neural tube defects.

In response to Dr Kittisupamongkol, glitazones were used by only a small number of our study participants (n=103 in the active treatment and n=120 in the placebo groups at 60 months of follow-up). Even if all these patients were taking rosiglitazone, it is unlikely that any effect of rosiglitazone on the risk of myocardial infarction and cardiovascular death in such a small subset of our patient population would have appreciably influenced the overall incidence of cardiovascular disease in the trial or altered the results related to our intervention.

Christine M. Albert, MD, MPH
calbert@partners.org
Nancy R. Cook, ScD
JoAnn E. Manson, MD, DrPH
Brigham and Women's Hospital
Boston, Massachusetts

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Chemotherapy and Radiotherapy for Anal Canal Carcinoma

To the Editor: Dr Ajani and colleagues[1] compared a combination of fluorouracil, mitomycin, and radiotherapy vs fluorouracil, cisplatin, and radiotherapy for the treatment of anal canal carcinoma. The 2 study groups had similar levels of toxicity, and the adherence to both chemotherapy and radiotherapy was comparable. However, there were some differences in treatment results.

One possible explanation for the significantly better time to colostomy in the standard treatment group is not linked to the chemotherapy itself, but rather to the immediate starting of radiotherapy. In the experimental group, radiation was delayed for almost 2 months. The importance of immediate combined treatment has been shown for laryngeal cancer[2] and may also be true for anal carcinoma, which is especially sensitive to radiotherapy.[3]

Daniela Musio, MD
Giovanni Codacci-Pisanelli, MD
oncologialatina@uniroma1.it
Department of Experimental Medicine
Università La Sapienza
Rome, Italy

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1. Ajani JA, Winter KA, Leonard L, et al. Fluorouracil, mitomycin, and radiotherapy vs fluorouracil, cisplatin, and radiotherapy for carcinoma of the anal canal: a randomized controlled trial. *JAMA*. 2008;299(16):1914-1921.

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BIOLOGY CONTRIBUTION

RECIPROCAL PARACRINE INTERACTIONS BETWEEN NORMAL HUMAN EPITHELIAL AND MESENCHYMAL CELLS PROTECT CELLULAR DNA FROM RADIATION-INDUCED DAMAGE

YUKA NAKAZAWA, M.S.,* VLADIMIR SAENKO, PH.D.,† TATIANA ROGOUNOVITCH, M.D.,* KEIJI SUZUKI, PH.D.,* NORISATO MITSUTAKE, M.D.,* MICHIKO MATSUSE, M.S.,* AND SHUNICHI YAMASHITA, M.D.*†

*Department of Molecular Medicine and †Department of International Health and Radiation Research, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

Purpose: To explore whether interactions between normal epithelial and mesenchymal cells can modulate the extent of radiation-induced DNA damage in one or both types of cells.

Methods and Materials: Human primary thyrocytes (PT), diploid fibroblasts BJ, MRC-5, and WI-38, normal human mammary epithelial cells (HMEC), and endothelial human umbilical cord vein endothelial cells (HUV-EC-C), cultured either individually or in co-cultures or after conditioned medium transfer, were irradiated with 0.25 to 5 Gy of γ-rays and assayed for the extent of DNA damage.

Results: The number of γ-H2AX foci in co-cultures of PT and BJ fibroblasts was approximately 25% lower than in individual cultures at 1 Gy in both types of cells. Reciprocal conditioned medium transfer to individual cultures before irradiation resulted in approximately a 35% reduction of the number γ-H2AX foci at 1 Gy in both types of cells, demonstrating the role of paracrine soluble factors. The DNA-protected state of cells was achieved within 15 min after conditioned medium transfer; it was reproducible and reciprocal in several lines of epithelial cells and fibroblasts, fibroblasts, and endothelial cells but not in epithelial and endothelial cells. Unlike normal cells, human epithelial cancer cells failed to establish DNA-protected states in fibroblasts and *vice versa*.

Conclusions: The results imply the existence of a network of reciprocal interactions between normal epithelial and some types of mesenchymal cells mediated by soluble factors that act in a paracrine manner to protect DNA from genotoxic stress. © 2008 Elsevier Inc.

Radiation, DNA damage, γ-H2AX, Epithelial–mesenchymal cell interaction, Paracrine factors.

INTRODUCTION

Radiation response is complex, and the intricacy further increases when effects are examined at the level of tissue. Developmentally every tissue accommodates different types of cells, for example, those of epithelial and mesenchymal origin in parenchymal organs. Tissue homeostasis is dynamically maintained by reciprocal endocrine and paracrine interactions between epithelium and stroma. However knowledge about the role of epithelial and stromal cell cooperation in radiation response is limited.

Several previous studies have addressed this issue. An increased clonogenicity and radioresistance of human head-and-neck squamous carcinoma cells have been observed when they were co-cultured with human fibroblasts, suggestive of a radioprotective effect of epithelial and stromal cell cooperation (1). Similarly human umbilical vein endothelial cells (HUVEC) co-cultured with U87 glioblastoma cells have been found to acquire resistance to radiation-induced apoptosis (2). In contrast, co-culturing of normal dermal fibroblasts with human breast cancer cell line MCF-7 in three-dimensional collagen gels resulted in radiosensitization of the latter (3). Co-culture of preirradiated autologous fibroblasts increased radiosensitivity of megacolonies of murine cervical squamous carcinoma cells (4) and also endothelial cells radiosensitized oropharyngeal squamous carcinoma cells in collagen gels (5). Together these data imply, although somewhat controversially, that interactions between epithelial and mesenchymal cells can modulate the effects of radiation exposure.

Reprint requests to: Vladimir Saenko, Ph.D., Department of International Health and Radiation Research, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. Tel: (+81) 95-819-7122; Fax: (+81) 95-819-7169; E-mail: saenko@net.nagasaki-u.ac.jp

Conflict of interest: none.

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Of note, the above-mentioned works have examined manifestations of radiation response in the context of interactions of mesenchymal cells with transformed epithelium, *i.e.*, in cancer treatment settings. Even less, if anything, is known about an ability of different types of normal cells to affect each other's capacity to cope with radiation. Damage to normal tissues inevitably occurs during radiation therapy, nuclear medicine procedures, occupationally in some professionals, and during involuntary exposure to the environment or accidents involving radiation. Therefore, understanding cross-talk between different types of normal cells exposed to radiation is important from biologic, medical, and public health viewpoints.

This study set out to investigate the reciprocal influence of different types of normal cells on the extent of radiation-induced DNA damage. More specifically, we used primary human thyrocytes and human mammary epithelial cells (HMEC) as representatives of epithelial tissues. The thyroid and breast are among the most radiation-sensitive organs in humans beings, as seen from the studies of carcinogenic effects of medical exposures, the atomic bombing of Japan (6, 7), and the Chernobyl accident (8–10). Several lines of human diploid fibroblasts and endothelial cells were tested as a cellular component of the stroma. DNA damage after irradiation was evaluated as a number of γ-H2AX–containing foci that, although not equivalent, correlate well with DSBs (11) and are commonly used for quantitative measurements. Cell co-culture and conditioned medium transfer were used to address the mechanisms of intercellular interactions.

METHODS AND MATERIALS

Cell lines

Primary human thyroid cell (PT) cultures were established as described previously (12) and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO):F12 (Sigma) (1:2) medium supplemented with 3.3% fetal bovine serum (FBS; Gibco, Grand Island, NY) and 1% penicillin/streptomycin (Sigma). These cultures contain at least 90% of epithelial cells according to cell morphology. In the course of this work, 11 independent PT cultures were used. Normal human foreskin diploid fibroblasts BJ (ATCC, Manassas, VA) and normal human fetal lung fibroblast lines MRC-5 and WI-38 (JCRB, Japan) were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1 mmol/l sodium pyruvate (Sigma), and 1X nonessential amino acids (Sigma) unless otherwise specified. The BJ, MRC-5, and WI-38 cells were used during passages 14 to 26, 19 to 24, and 16 to 21, respectively. A normal human endothelial cell strain, HUV-EC-C (HSRRB, Japan), was maintained in EGM-2 medium (Cambrex, Walkersville, MD) supplemented with 2% fetal bovine serum and 1% penicillin/streptomycin; cells were used during passages 31 to 35. Normal HMEC (Cambrex) were cultured in HEGM-2 medium (Cambrex) and used during passages 9 to 10. Human anaplastic thyroid carcinoma cell lines ARO and FRO, papillary thyroid carcinoma cell lines NPA and TPC-1, and breast cancer cell line MCF-7 were maintained in RPMI-1640 medium supplemented with 5% FBS and 1% penicillin/streptomycin. All cell cultures in this study were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Individual cell cultures and co-cultures

In monocultures, cells (1×10^5/well) were seeded onto sterilized glass coverslips in the 12-well plates, yielding 100% confluent cultures. In co-cultures, PT and BJ cells were mixed at a ratio of 1:1 and cultured in the medium used for fibroblasts or PT. To discriminate between different types of cells (*i.e.*, PT and BJ) under a fluorescent microscope in co-cultures, BJ cells were prelabeled with 500 μmol/l Mito Tracker Red 580 (Molecular Probes, Invitrogen, Eugene, OR) for 15 min at 37°C. Individually or co-cultured cells were incubated overnight before irradiation or other treatments.

Conditioned medium transfer

Medium was conditioned on confluent cultures of each cell line overnight. The next day recipient cells were washed with phosphate-buffered saline (PBS), and conditioned medium (CM) was added after filtration though a 0.22-μm sterile syringe filter or intact. (Filtration or centrifugation of the conditioned medium at 16,000 *g* for 10 min at room temperature did not significantly change results; data not shown.) After CM transfer, cells were incubated for different periods before irradiation, as described later here.

Irradiation and immunofluorescence

Cells grown on coverslips were exposed to a single acute dose of 0 to 5 Gy of γ-rays from a ^{137}Cs source (PS-3100SB, Pony, Japan) at a dose-rate of 1 Gy/min and incubated at 37°C for 30 min unless otherwise specified. Fixation and immunostaining with the monoclonal anti-phosphorylated histone H2AX (Ser139) antibody (Upstate Biotechnology, Lake Placid, NY), anti-phosphorylated ATM (Ser1981) (Rockland, Gilbertsville, PA), anti-53BP1 (Bethyl Laboratories, Montgomery, TX) or antireplication protein A (RPA) (Oncogene Research Products, San Diego, CA) were done as described (13). After incubation with Alexa Fluor 488-labeled antimouse IgG (Molecular Probes, Eugene, OR) in the presence of 10 μg/ml RNAse A (Qiagen, Hilden, Germany), cover slips were washed with PBS, stained with TO-PRO-3 iodide (Invitrogen) and mounted with GEL/MOUNT medium (Biomeda, Foster City, CA). The images were captured with a LSM510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany). γ-H2AX foci in at least 100 nonoverlapping nuclei were automatically counted for each data point using the Count/Size procedure of the Image-Pro Plus version 4.5 software package (Media Cybernetics, Inc., Bethesda, MD). Details of parameter settings are available from the authors upon request.

Medium treatment with lindane, heating, and trypsin

The PT cells and BJ fibroblasts were incubated in individual cultures or in co-cultures for 6 h, after which the medium was supplemented with 50 μmol/l lindane (1,2,3,4,5,6-hexachlorocyclohexane, Wako, Japan) or DMSO as a vehicle (14), and the cultures were incubated for additional 18 h before irradiation. The efficacy of gap junction intercellular communication (GJIC) blockage was controlled using scrape-loading/dye transfer technique (15); data not shown.

For heat inactivation experiments, CM from PT and BJ cultures was incubated at 37°C or 56°C for 30 min and transferred to recipient cells which were then irradiated with 1 Gy of γ-rays 1 h later.

For proteolytic treatment of CM, plain DMEM conditioned overnight on confluent cells was used. In our preliminary experiments we found that both PT and BJ cells tolerate unsupplemented DMEM during 24 h without changes in cell morphology. A 1-ml quantity of conditioned DMEM was incubated at 37 °C for 3 h under mild swirling with 130 to 140 mg of PBS-washed and minicolumn-spun BSA beads (made by coupling the protein to BrCN-activated Sepharose 4B, Amersham Biosciences, Stockholm, Sweden) or

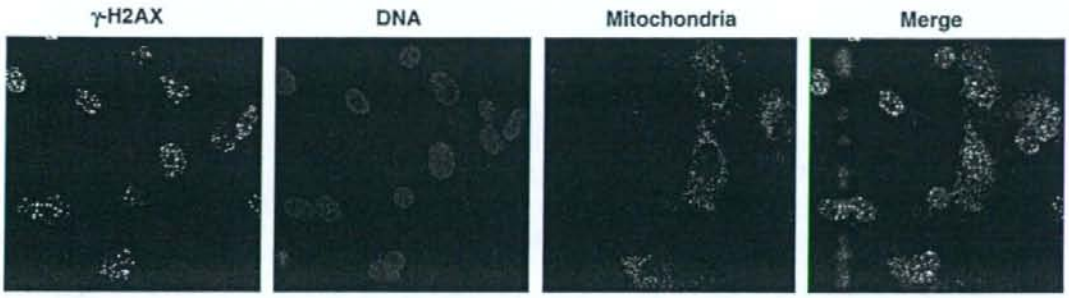

Fig. 1. Detection of nuclear γ-H2AX foci in co-culture of BJ fibroblasts and primary thyrocytes (PT). Cells were irradiated with 1Gy of γ-rays and fixed 30 min after exposure. The BJ cells were prestained with a mitochondria tracking dye (red). Magnification ×60.

TPCK-Trypsin beads (Pierce, Rockford, IL). Beads were sedimented by centrifugation and clarified medium was transferred to recipient cells. Irradiation with 1 Gy of γ-rays was performed 1 h later.

Comet assay

The PT and BJ cells were plated on 60-mm dishes at ~100% confluence, exposed to the medium conditioned on homologous or heterologous cells for 1 h and irradiated with 1 Gy of γ-rays. Immediately after irradiation the cells were washed with PBS and collected by scraping with a rubber policeman. The assay was done with a Comet Assay Kit (Trevigen, Gaithersburg, MD) according to the manufacturer's instructions for alkaline conditions. Briefly, electrophoresis was performed for 30 min (1 V/cm, 250 mA), then the slides were washed, immersed in 70% ethanol for 5 min, air dried, and stained with SYBR Green I dye. Images were captured using an Eclipse TE2000-U fluorescent microscope (Nikon, Japan) equipped with CCD camera. At least 200 cells were analyzed using CometScore software (TriTek Corp., Morrisville, VA).

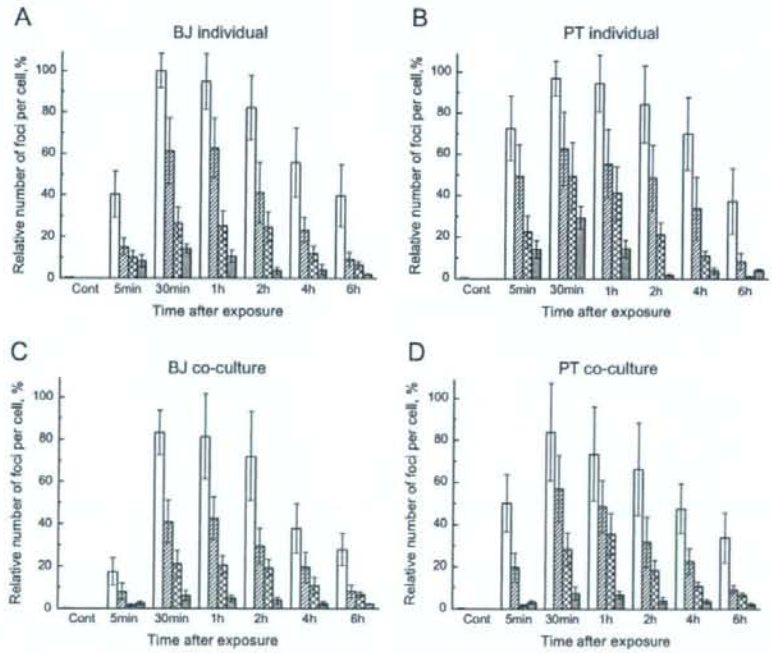

Fig. 2. The relative number of γ-H2AX foci in BJ and PT cells in individual cultures (a and b, respectively) and in co-cultures (c and d, respectively) at various time points after irradiation. Open bars correspond to dose of 5 Gy; hatched bar, 2 Gy; cross-haired bar, 1 Gy; filled bar, 0.5 Gy. Data are shown as the mean ± SD. The Y-axis represents the number of γ-H2AX foci in relation to the number of foci detected 30 min after exposure of BJ fibroblasts to 5 Gy of γ-rays (100%) for simplicity. Note that direct comparison of the numbers of foci between different cultures shown on this graph would be inappropriate as data were obtained in different experiments. Estimated average number of foci in nonirradiated cultures was 0.15 per cell. Results are representative of two independent experiments.

Statistical analysis

Significance of difference among datasets was assessed by t-test or ANOVA followed by Tukey's multiple comparison *post hoc* test (for $p < 0.05$) as appropriate. A p value of < 0.05 was considered statistically significant.

RESULTS

γ-H2AX foci number in irradiated individually cultured and co-cultured PT and BJ cells

To distinguish different types of cells in co-cultures, fibroblasts were labeled with a mitochondria-specific tracker dye (Fig. 1). The number of γ-H2AX foci rapidly increased in irradiated cells reaching a peak at 30 min to 1 h in a dose-dependent manner (Fig. 2) and then gradually decreased to return to the control level by 24 h (data not shown). Temporal changes in the number of foci were similar in both individually and co-cultured cells demonstrating that DNA repair in them was equally effective. In view of these results, cells were fixed 30 min after exposure in further experiments.

As shown in Fig. 3, the relative number of γ-H2AX foci in PT/BJ co-cultures was significantly lower (~25% less) than that in individual cultures in both types of cells at all radiation doses tested (Figs. 3A, 3B). A rather smooth shift towards the lower foci number was suggestive of the generally uniform diminution of radiation-induced DNA damage in co-cultures as compared with individual cultures (Figs. 3C, 3D).

Role of gap junction intercellular communication

To address the involvement of GJIC, this type of cell-to-cell interaction was blocked with a noncytotoxic dose of lindane. The presence of the chemical in medium led to an increase in the number of γ-H2AX foci in PT and BJ cells, in both individual cultures and co-cultures (Fig. 4). However, despite the increase, the number of γ-H2AX foci in any type of cells in PT/BJ co-culture did not reach values observed in lindane-treated individual cultures. These results imply that functional GJIC can partly affect (*i.e.*, diminish) the extent of radiation-induced DNA damage at relatively high radiation doses, but that it is unlikely to be involved in the diminished DNA damage (DDD) effect arising because of cell co-culturing.

Involvement of soluble factors in the attenuation of radiation-induced DNA damage

The effect of soluble factors was examined using CM transfer methodology. Individual PT or BJ cultures were

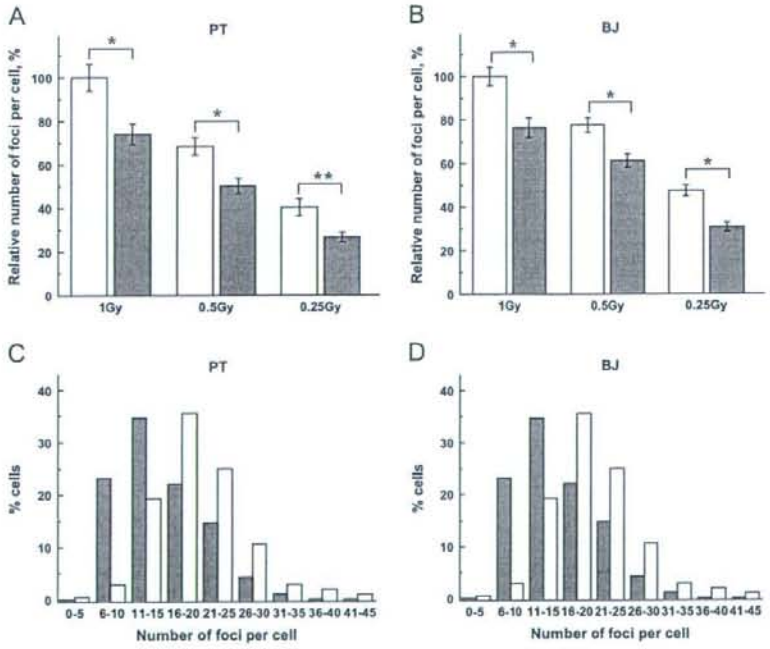

Fig. 3. The relative number of γ-H2AX foci in primary thyrocytes (PT) (A) and BJ (B) cells in individual cultures (open bar) and co-cultures (filled bar) 30 min after irradiation with different doses of γ-rays. The number of foci in individual PT (C) (19.73 foci/cell, average) and BJ (D) (24.27 foci/cell, average) cultures after irradiation with 1 Gy was assigned 100% on respective graphs. Data were pooled from three independent experiments in which cells were co-cultures in fibroblast medium. Data are shown as the mean ± SE unless otherwise specified. Asterisks depict statistically significant values (* $p < 0.001$, ** $p < 0.01$). Similar data were obtained when cells were co-cultured in PT medium (data not shown). (C, D) Frequency histogram of foci number in individual cultures (open bar) and co-cultures (filled bar) irradiated with 1 Gy of γ-rays.

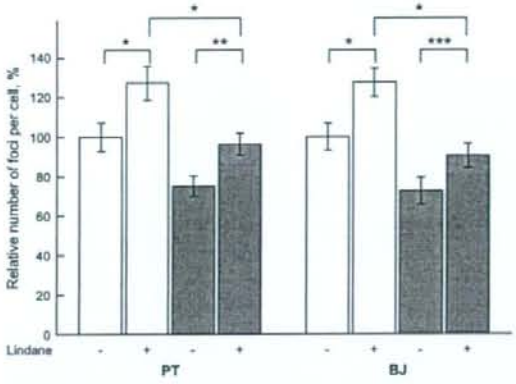

Fig. 4. Effect of gap junction intercellular communication blockage on the number of radiation-induced γ-H2AX foci. The primary thyrocytes (PT) cells and BJ fibroblasts were incubated in individual cultures (open bars) or in co-cultures (filled bars) with lindane or vehicle added. The number of foci in individual PT and BJ cultures after irradiation with 1 Gy of γ-rays was assigned 100%. Data were pooled from three independent experiments and are shown as the mean ± SE. Asterisks depict values that are statistically significant (*$p < 0.001$, ** $p < 0.01$, *** $p < 0.05$). Similar results were obtained in cells irradiated with 0.25 and 0.5 Gy (data not shown).

incubated with the medium conditioned on homologous or heterologous cells (homo-CM and hetero-CM, respectively) or with fresh heterologous cell medium and irradiated. The number of γ-H2AX foci in both cell types appeared to be significantly lower in the cells that received hetero-CM (Fig. 5A). In CM transfer experiments, the DDD effect was more pronounced than in co-cultures (~35% and ~25% decrease in the number of foci, respectively). No significant changes in the number of foci were observed after cell incubation with fresh heterologous medium, ruling out influence of medium components. In addition, immunofluorescent staining for 53BP1 and phospho-ATM, which, like γ-H2AX, also are markers of DNA damage, demonstrated perfect co-localization of all three proteins in the nuclear foci in both PT and BJ cells after irradiation (Fig. 5B).

Because alterations in growth factors in CM might stimulate cell progression to S-phase in which cells form γ-H2AX foci independently of irradiation (16) or may display a double number of nuclear foci after exposure (13), we determined the frequency of cells positive for RPA (an S-phase cell marker) in routinely used confluent cultures and at 50% confluence. The PT cultures harbored a very low number of S-phase cells at any cell density (Fig. 5C), whereas a significant number of those was observed in the loosely plated fibroblasts. Importantly, in both types of cells, CM transfer did not change the proportion of RPA-positive cells.

To this point, preincubation of target cells with CM was done overnight before irradiation. We next questioned how rapidly DDD effect takes place after medium transfer, and exposed cells to hetero-CM for 5 min to 2 h before irradiation.

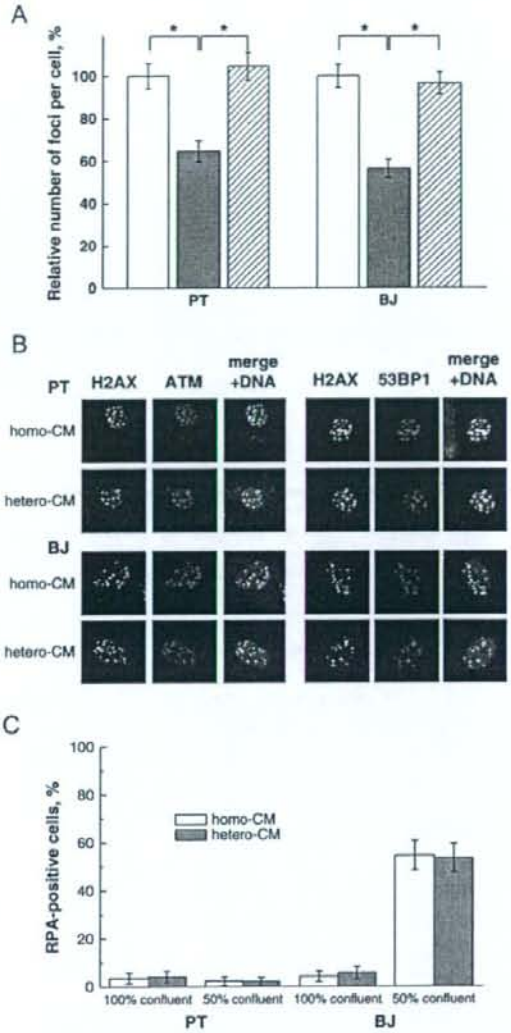

Fig. 5. (A) Effect of conditioned medium transfer (overnight preincubation) on the number of γ-H2AX foci in irradiated (1 Gy) primary thyrocytes (PT) and BJ cells. Recipient cells are indicated below the horizontal axis. Open bars correspond to homo-CM; filled bars, hetero-CM; hatched bar, fresh heterologous cell medium. The number of foci in cell cultures that received homo-CM was assigned 100%. Data were pooled from three independent experiments and are shown as the mean ± SE. * $p < 0.001$. (B) Co-localization of γ-H2AX, phospho-ATM and 53BP1 foci after homo-CM and hetero-CM transfer in the cells irradiated with 1 Gy. (C) Frequency of RPA-positive cells in 100% and 50% confluent cultures of PT and BJ cells treated with homo-CM and hetero-CM (mean ± SD).

Unexpectedly, even a very short incubation with hetero-CM was sufficient to elicit DDD. A full response was achieved during 15 min after hetero-CM transfer, and then it remained unchanged for the time interval tested (Fig. 6A). Because of