RAPID COMMUNICATION Jpn. J. Cancer Res. 87, 1111–1115, November 1996

Increased Telomerase Activity in Hyperplastic Nodules and Hepatocellular Carcinomas Induced by a Choline-deficient L-Amino Acid-defined Diet in Rats

Toshifumi Tsujiuchi, Masahiro Tsutsumi, Akira Kido, Kunihiko Kobitsu, Makoto Takahama, Toshimitsu Majima, Ayumi Denda, Dai Nakae and Yoichi Konishi[1]

Department of Oncological Pathology, Cancer Center, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634

Activation of telomerase has been reported in several human cancers, including hepatocellular carcinomas (HCCs). We investigated telomerase activity during hepatocarcinogenesis induced by a choline-deficient L-amino acid-defined (CDAA) diet in rats. Male F344 rats were given a CDAA diet or a choline-supplemented L-amino acid-defined (CSAA) diet from 6 weeks of age for 75 weeks, and subgroups were killed 10 weeks, 50 weeks and 75 weeks after the beginning of the experiment. Hyperplastic nodules and HCCs were noted in rats fed a CDAA diet for 50 weeks and 75 weeks, respectively. Normal control liver specimens were obtained from 6-week-old rats. Telomerase activity was assessed by using a telomeric repeat amplification protocol (TRAP). Normal liver and background parenchyma of rats fed either of the diets for 10 weeks or 50 weeks showed weak telomerase activity. In contrast, markedly increased levels were demonstrated in hyperplastic nodules and HCCs. These results suggest that increased telomerase activity may be a biological feature of preneoplastic lesions that evolve to HCCs in rat liver.

Key words: Choline-deficient L-amino acid-defined diet — Hepatocarcinogenesis — Telomerase — TRAP assay — Rat

Neoplasia consists of multiple qualitatively different steps in which accumulation of DNA alterations occurs and critical events, such as the modifications of APC, Ki-*ras* and *p53* genes, take place, as reported for human colon carcinogenesis.[1,2] Recently, it has been hypothesized by Kim *et al.*[3] that activation of telomerase is necessary for tumor cells to achieve immortality and proceed to the malignant state. In man, although activation of telomerase is not detectable in normal tissues except for germ cells, lymphocytes, hematopoietic progenitor cells and epidermis,[3–6] malignant tumors, including HCCs,[2] show a high activity which is not shown by non-tumorous lesions or benign counterparts.[3,4,7–12] Therefore, it has been suggested that activation of this enzyme might be a critical factor defining the onset of the malignant state during carcinogenesis. In contrast to the human case, normal tissues in mice and rats, including the liver, show telomerase activity.[13–15] The relative ease of immortalization of murine primary cells, as compared to human cells, might be due to this difference in telomerase.[14]

Recently, it has been reported that telomerase activity increases with malignant progression during skin carcinogenesis in mice, though it is undetectable in normal skin.[16] To develop an understanding of the biological roles of telomerase, especially with reference to the preneoplasia and neoplasia link, investigations of the species or strain dependence of its expression are necessary. However, so far no reports have been published on the relation of telomerase to liver carcinogenesis in rats. Recently we established a rat hepatocarcinogenesis model using a CDAA diet, which is useful for studies of endogenous hepatocarcinogenesis, possibly mediated by oxidative DNA damage, without carcinogen application.[17,18] The aim of the present experiment was to identify changes in telomerase activity during CDAA-hepatocarcinogenesis, with HCCs as the endpoint.

Male F344 rats (Shizuoka Laboratory Animal Center, Shizuoka), 6 weeks old at the commencement, were given a CDAA or CSAA diet (product number 518753 or 518754, respectively; Dyets Inc., Bethlehem, PA), the compositions of which were described previously.[17,18] Subgroups of 3 to 5 rats were killed at 10, 50 and 75 weeks after the beginning of the experiment, and 3 rats aged 6 weeks were similarly killed under ether anesthesia to obtain normal control livers. The livers were immediately removed and frozen in liquid nitrogen in their entirety in the control and 10-week cases. Unequivocal

[1] To whom correspondence should be addressed.

Abbreviations: HCCs, hepatocellular carcinomas; CDAA diet, choline-deficient L-amino acid-defined diet; CSAA diet, choline-supplemented L-amino acid-defined diet; TRAP, telomeric repeat amplification protocol.

liver nodules and cancers of rats killed at 50 and 75 weeks were separated from non-tumorous areas and similarly frozen and stored at −80°C. Portions of each liver tissue including nodules, cancers and non-tumorous areas were also fixed in formalin, routinely processed and stained with hematoxylin and eosin (HE) for histological examination.

Tissue extracts were prepared with the procedures described previously[3, 16] with some modifications. Briefly, frozen tissue was rinsed in cold buffer [23 m*M* HEPES (pH 7.5), 6.9 m*M* KCl, 2.3 m*M* $MgCl_2$, 2.3 m*M* DTT, 0.23 m*M* PMSF, 2 U/ml RNA guard (Pharmacia, Piscataway, NJ), 2.3 μM leupeptin and 23 μM pepstatin A)], and incubated on ice for 10 min, then homogenized by hand with a Teflon pestle. After incubation for 30 min, the samples were centrifuged for 10 min at 12000 rpm at 4°C. A one-fiftieth volume of 5 *M* NaCl was then added and the samples were again centrifuged for 1 h at 100,000*g* at 4°C. The resultant supernatants were stored at −80°C until assayed for telomerase activity. Protein concentrations were determined by the DC protein assay (Bio Rad, Richmond, CA).

The telomerase assay was performed by a modification of the procedure described previously.[3, 16] Fifty microliters of a mixture containing PCR buffer [30 m*M* Tris-Cl (pH 8.3), 1.5 m*M* $MgCl_2$, 68 m*M* KCl, 5 m*M* BME, 0.5 m*M* EGTA, 0.05% NP-40 and 0.05% Tween 20], 0.1 μg of TS primer (5′-AATCCGTCGAGCAGAGTT-3′), 50 μM dNTPs, 1 μg of T4g32 protein (Boehringer-Mannheim, Mannheim), 3 units of Taq DNA polymerase (Pharmacia, Milwaukee, WI), 0.4 μl of [α-^{32}P]-dCTP (3000 Ci/mmol) and 5 μg of extract was incubated for 30 min at 25°C for extension of the TS primer by any included telomerase. The mixture was then heated to 90°C for 3 min and 0.1 μg of CX primer (5′-CCCTTACCCTTACCCTTACCCTAA-3′) was added. The PCR procedure was performed with 31 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 45 s, followed by 72°C for 8 min. To determine the sensitivity to RNase, some samples were incubated with 1 μl of RNase A (1 mg/ml) for 30 min at 37°C, and used for the TRAP assay. For negative controls, mixtures without TS primer or CX primer were also included. Fifteen microliters of the PCR products was electrophoresed on 10% non-denaturing polyacrylamide gels. Autoradiographs were quantitated with a BAS 1000 image analyzer (Fuji Photo Film Co., Ltd., Tokyo). The signal intensities of three bands from the lower levels of the gels, excepting the bottom band, were measured and the sum of these was compared with that for normal liver, simultaneously electrophoresed in each experiment. The background in the negative control lanes was subtracted from all signal intensity values for experimental samples.[16]

Histologically, the livers of rats fed a CDAA diet for 10 weeks demonstrated fatty cirrhosis with putative preneoplastic foci and after 50 weeks hyperplastic nodules, easily recognized in HE-stained sections and showing γ-glutamyltransferase (GGT) activity, became apparent. At the 75 week time point, well-differentiated HCCs were observed with a background of cirrhosis. The infiltration of lymphocytes was not seen in hyperplastic nodules or HCCs.

To assess the methodology for the determination of the activity, we modified the methods of protein extraction and the PCR conditions documented by Kim *et al.*[3] and Bednarek *et al.*[16] for human and mouse tissues, respectively. The results of incubation time- and extract dilution-dependent assays showed that 6 bp ladders could be

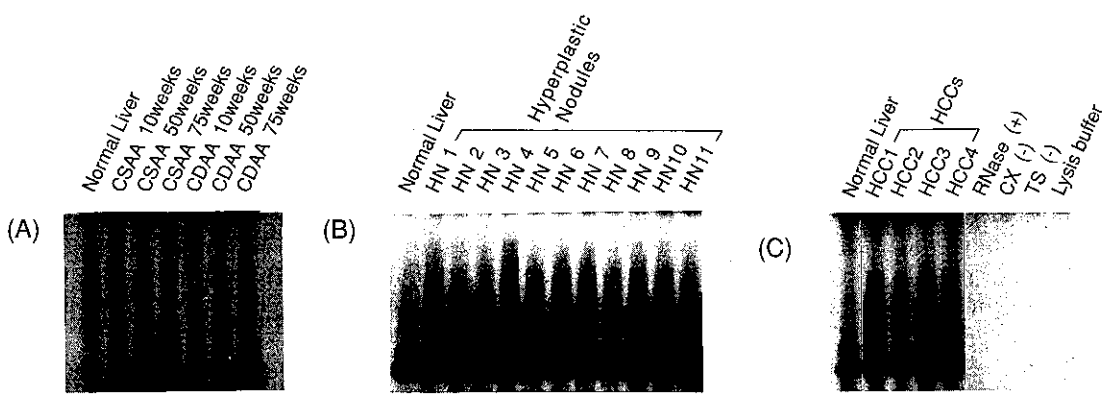

Fig. 1. TRAP assay for telomerase activity. (A) Results for normal liver and non-cancerous tissues from rats receiving CSAA or CDAA diets for 10, 50 and 75 weeks. (B) Results for normal liver and hyperplastic nodules induced by administration of a CDAA diet for 50 weeks. HN, hyperplastic nodule. (C) Results for normal liver and HCCs induced by administration of a CDAA diet for 75 weeks. RNase(+), HCC1 extract treated with RNaseA; CX(−), HCC1 extract without CX primer; TS (−), HCC1 extract without TS primer; Lysis buffer, Lysis buffer and primer without tissue extract.

Table I. Densitometric Quantification of Relative Telomerase Activities

Samples	Densitometric quantification (P-B)[a]/mm²	Telomerase activity relative to normal liver[b]
(A)		
Normal liver	17.4	1.00
CSAA 10 wks	20.7	1.19
CSAA 50 wks	25.4	1.46
CSAA 75 wks	23.6	1.36
CDAA 10 wks	20.4	1.17
CDAA 50 wks	22.3	1.28
CDAA 75 wks	23.4	1.34
(B)		
Normal liver	25.6	1.00
HN 1	110.1	4.30
HN 2	132.2	5.16
HN 3	136.0	5.31
HN 4	176.0	6.88
HN 5	125.0	4.88
HN 6	154.9	6.05
HN 7	119.4	4.66
HN 8	96.2	3.76
HN 9	102.2	3.99
HN 10	100.6	3.93
HN 11	84.4	3.30
(C)		
Normal liver	20.5	1.00
HCC 1	129.8	6.33
HCC 2	91.6	4.47
HCC 3	123.5	6.02
HCC 4	100.2	4.89

a) P-B: Photostimulating luminescence-background.
b) Ratios of telomerase activities to the normal liver control value.

detected at a concentration of 5 μg protein for 30 min at 25°C. When the extracted samples were diluted 10- to 100-fold, the differences in telomerase activity between HCCs and normal livers were still detected as ladders obtained from non-diluted tissue extracts (data not shown), confirming the method modified by ourselves to be useful for rat liver. The samples with RNase, without TS primer or CX primer, and the sample without extracted tissue showed no ladders.

Our TRAP assay data are summarized in Fig. 1 and Table I. Telomerase activity in normal liver and in livers of animals fed the CSAA diet until 75 weeks and the CDAA diet-fed rats at 10 weeks did not exhibit any significant elevation. Increased activity could, however, be clearly detected in all hyperplastic nodules and HCCs compared with normal liver.

Recently, high frequencies of increased telomerase activities have been noted in various human malignancies, such as carcinomas of the lung,[7] breast,[3] prostate,[3, 10] colon,[3, 11] liver,[3, 9] brain[3, 12] and ovaries,[8] as well as lymphomas,[3] Wilm's tumors,[3] head and neck tumors,[3] rhabdomyosarcomas[3] and leiomyosarcomas,[3] but not in normal or background tissues, or benign lesions related to the above malignancies. Telomerase is not detectable in most human adult tissues other than the germ cells, lymphocytes, hematopoietic progenitor cells and epidermis.[3–6] Human liver was reported to show no telomerase activity,[3, 4] but activated telomerase has been detected in the liver of mice and rats.[13–15] The relevance of the differences between humans, mice and rats to the biological function of telomerase activation in normal tissues remains to be clarified.

In this study, hyperplastic nodules showed markedly increased telomerase activities. Bednarek *et al.*[16] earlier reported a progressive increase from early papillomas to late papillomas in an initiation-promotion skin carcinogenesis model in mice and speculated that this was associated with an increased level of genomic instability and phenotypic progression. Hyperplastic nodules are generally accepted as precusor lesions which can progress to HCCs, although it has been pointed out that some are reversible.[19] We examined 11 apparently irreversible nodules which were 3 to 5 mm in diameter and demonstrated diffuse positive GGT activity, and detected increased telomerase in all of them. Therefore, the results are consistent with those for mouse skin papillomas in pointing to a role for achievement of immortality before progression to carcinomas during carcinogenesis. The foci present in the livers of rats fed the CDAA diet for 10 weeks did not show increased telomerase activity, suggesting that these lesions are reversible and consist of mortal cells. It has, however, been reported that hepatocyte foci of rats fed the carcinogen 2-acetylaminofluorene for more than 9 weeks can be cultured *in vitro*, and become transplantable on being promoted by phenobarbital.[20]

Telomere reduction has been reported with aging of human fibroblasts[21, 22] and in colorectal,[23] ovarian,[8] renal cell[24] and hepatocellular carcinomas.[25] Furthermore, correlations between shortened telomere length and elevated telomerase activities have been demonstrated in HCCs[9, 25] and ovarian carcinomas,[8] suggesting a need for increased enzyme expression for chromosome stabilization and immortality *per se*. In this context, it would clearly be of interest to determine telomere lengths in hyperplastic nodules and HCCs.

In conclusion, the increased telomerase activity evident in hyperplastic nodules and HCCs induced by a CDAA diet in the present study provides support for the view that activation of this enzyme is required for preneoplastic cells to attain immortality and progress to neoplasia. Recently, strategies to inhibit telomerase as an approach to cancer therapy have been highlighted,[26] but basic information is still needed from molecular and immunological studies. Experimental work *in vivo*, tar-

geting the role of telomerase activation in carcinogenesis, should lead to an understanding of how transformation of preneoplastic lesions to malignancies can be prevented.

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, and a Grant for Scientific Research Expenses in the Health and Welfare Program from the Ministry of Health and Welfare, Japan. We would like to thank Drs. E. Tahara and H. Tahara for helpful discussions, and Kazue Ichida and Rie Maeda for their assistance in the preparation of the manuscript.

(Received July 10, 1996/Accepted August 27, 1996)

REFERENCES

1) Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., Whyte, R., Smits, A. M. M. and Bos, J. L. Genetic alterations during colorectal tumor development. *N. Engl. J. Med.*, **319**, 525–532 (1988).
2) Miyaki, T., Seki, M., Okamoto, M., Yamanaka, A., Maeda, Y., Tanaka, K., Kikuchi, R., Iwama, T., Ikeuchi, T., Tonomura, A., Nakamura, Y., White, R., Miki, Y., Utsunomiya, J. and Koike, M. Genetic changes and histopathological types in colorectal tumors from patients with familial adenomatous polyposis. *Cancer Res.*, **50**, 7166–7173 (1990).
3) Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L. C., Coviello, G. H., Wright, W. E., Weinrich, S. L. and Shay, J. W. Specific association of human telomerase activity with immortal cells and cancer. *Science*, **266**, 2011–2015 (1994).
4) Harley, C. B., Andrews, W., Chiu, C.-P., Feng, J., Funk, W., Gaeta, F., Hirsch, K., Kim, N. W., Kozlowski, M., Wang, S.-S., Weinrich, S. L., West, M. D., Avillion, A., Le, S., Greider, C. W. and Villeponteau, B. Human telomerase inhibition and cancer. *Proc. Am. Assoc. Cancer Res.*, **36**, 671–672 (1995).
5) Hiyama, K., Hirai, Y., Kyoizumi, S., Akiyama, H., Hiyama, E., Piatyszek, M. A., Shay, J. W., Ishioka, S. and Yamakido, M. Activation of telomerase in human lymphocytes. *J. Immunol.*, **155**, 3711–3715 (1995).
6) Taylor, R. S., Ramirez, R. D., Ogushi, M., Chaffins, M., Piatyszek, M. A., Shay, J. W. Detection of telomerase activity in malignant and nonmalignant skin conditions. *J. Invest. Dermatol.*, **106**, 759–765 (1996).
7) Hiyama, K., Hiyama, E., Ishioka, S., Yamakido, M., Inai, K., Gazdar, A. F., Piatyszek, M. A. and Shay, J. W. Telomerase activity in small-cell and non- small-cell lung cancers. *J. Natl. Cancer Inst.*, **87**, 895–902 (1995).
8) Counter, C. M., Hirte, H. W., Bacchetti, S., Harley, C. B. Telomerase activity in human ovarian carcinoma. *Proc. Natl. Acad. Sci. USA*, **91**, 2900–2904 (1994).
9) Tahara, H., Nakanishi, T., Kitamoto, M., Nakashio, R., Shay, J. W., Tahara, E., Kajiyama, G. and Ide, T. Telomerase activity in human liver tissues: comparison between chronic liver disease and hepatocellular carcinomas. *Cancer Res.*, **55**, 2734–2736 (1995).
10) Sommerfeld, H.-J., Meeker, A. K., Piatyszek, M. A., Bova, G. S., Shay, J.W., Coffey, D. S. Telomerase activity: a prevalent marker of malignant human prostate tissue. *Cancer Res.*, **56**, 218–222 (1996).
11) Chadeneau, C., Hay, K., Hirte, H. W., Gallinger, S., Bacchetti, S. Telomerase activity associated with acquisition of malignancy in human colorectal cancer. *Cancer Res.*, **55**, 2533–2536 (1995).
12) Langford, L. A., Piatyszek, M. A., Xu, R., Schold, S. C. Jr., Shay, J. W. Telomerase activity in human brain tumors. *Lancet*, **346**, 1267–1268 (1995).
13) Bacchetti, S., Counter, C. M., Chadeneau, K. C., Gupta, J., Harley, C. B., Leber, B., Gallinger, S., Hirte, H. W., Siegel, P. and Muller, W. J. Telomerase activity in human and murine somatic tissues and tumors. *Proc. Am. Assoc. Cancer Res.*, **36**, 674 (1995).
14) Prowse, K. R., Greider, C. W. Developmental and tissue-specific regulation of mouse telomerase and telomere length. *Proc. Natl. Acad. Sci. USA*, **92**, 4818–4822 (1995).
15) Yoshimi, N., Ino, N., Suzui, M., Hara, A., Nakatani, K., Sato, S. and Mori, H. Telomerase activity of normal tissues and neoplasms in rat colon carcinogenesis induced by methylazoxymethanol acetate and its difference from that of human colonic tissues. *Mol. Carcinog.*, **16**, 1–5 (1996).
16) Bednarek, A., Budunova, I., Slaga, T. J., Aldaz, C. M. Increased telomerase activity in mouse skin premalignant progression. *Cancer Res.*, **55**, 4566–4569 (1995).
17) Nakae, D., Yoshiji, H., Maruyama, H., Kinugasa, T., Denda, A., Konishi, Y. Production of both 8-hydroxydeoxyguanosine in liver DNA and γ-glutamyltransferase-positive hepatocellular lesions in rats given a choline-deficient, L-amino acid-defined diet. *Jpn. J. Cancer Res.*, **81**, 1081–1084 (1990).
18) Nakae, D., Yoshiji, H., Mizumoto, Y., Horiguchi, K., Shiraiwa, K., Tamura, K., Denda, A. and Konishi, Y. High incidence of hepatocellular carcinomas induced by a choline deficient L-amino acid defined diet in rats. *Cancer Res.*, **52**, 5042–5045 (1992).
19) Enomoto, K., Farber, E. Kinetics of phenotypic maturation of remodeling of hyperplastic nodules during liver carcinogenesis. *Cancer Res.*, **42**, 2330–2335 (1982).
20) Kitagawa, T., Watanabe, R., Kayano, T., Sugano, H. *In vitro* carcinogenesis of hepatocytes obtained from acetylaminofluorene-treated rat liver and promotion of their growth by phenobarbital. *Gann*, **71**, 747–754 (1980).
21) Counter, C. M., Avilion, A. A., LeFeuvre, C. E., Stewart, N. G., Greider, C. W., Harley, C. B. and Bacchetti, S. Telomere shortening associated with chromosome instabil-

ity is arrested in immortal cells which express telomerase activity. *EMBO J.*, **11**, 1921–1929 (1992).
22) Harley, C. B., Futcher, A. B., Greider, C. W. Telomeres shorten during aging of human fibroblasts. *Nature*, **345**, 458–460 (1990).
23) Hastie, N. D., Dempster, M. G., Dunlop, M. G., Thompson, A. M., Green, D. K., Allshire, R. C. Telomere reduction in human colorectal carcinoma and with aging. *Nature*, **346**, 866–868 (1990).
24) Mehle, C., Ljungberg, B., Roos, G. Telomere shortening in renal cell carcinoma. *Cancer Res.*, **54**, 236–241 (1994).
25) Ohashi, K., Tsutsumi, M., Nakajima, Y., Kobitsu, K., Nakano, H., Konishi, Y. Telomere changes in human hepatocellular carcinomas and hepatitis virus infected noncancerous liver. *Cancer*, **77**, 1747–1751 (1996).
26) Rhyu, M. S. Telomeres, telomerase, and immortality. *J. Natl. Cancer Inst.*, **87**, 884–894 (1995).