Jpn. J. Cancer Res. **89**, 1074–1081, October 1998

Telomerase Activity Correlates with Growth of Transplantable Osteosarcomas in Rats Treated with *cis*-Diammine Dichloroplatinum or the Angiogenesis Inhibitor AGM-1470

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To determine the role of telomerase activity in the growth of tumors in rats undergoing chemotherapy, a comparison of the volumes of telomerase-positive transplantable osteosarcomas was made in rats treated with the antineoplastic agent *cis*-diammine dichloroplatinum (CDDP) or the angiogenesis inhibitor O-(chloroacetylcarbamoyl)fumagillol (AGM-1470). Male F344 rats, 8 weeks old, received transplants of macroscopic lung metastatic nodules into the subcutaneous back space and treatment was started on day 14 thereafter. CDDP was injected i.v. at doses of 0, 0.625, 1.25 and 2.5 mg/kg body weight (b.w.) and AGM-1470 was administered at total doses of 0, 2.5, 5 and 10 mg/kg b.w. over 2 weeks by osmotic pumps, also implanted into the subcutaneous back space, but remote from the transplanted tumors. On day 28, all animals were killed for measurement of transplanted tumor size and determination of telomerase activities by telomeric repeat amplification protocol (TRAP) assay. The results showed telomerase activity to be highly correlated with the treated/non-treated (T/C) tumor size ratio (r=0.96, P<0.0001). In a second experiment, CDDP at 2.5 mg/kg b.w. and AGM-1470 at 10 mg/kg b.w., these being the most effective doses, were given as in the first experiment, and animals were serially killed on days 14, 21, 28, 35 and 42. Tumors in rats treated with CDDP and AGM-1470 showed 18.2% and 20.5% of the control telomerase activity on days 35 and 21, respectively, when tumor growth was inhibited. However, on day 42, the activities increased to 46.5% and 92.5%, this correlating with re-growth (r=0.73, P<0.0001). These results suggest that decline of telomerase activity may be involved in tumor growth retardation induced by chemotherapeutic agents. This possibility clearly warrants further mechanistic studies.

Key words: Rat transplantable osteosarcoma — Chemotherapy — AGM-1470 — T/C rate — TRAP assay

Telomerase is an enzyme that contains an RNA complementary to the short DNA sequence repeats (GGTTAG in humans and rodents) located at chromosomal ends. The enzyme is believed to be involved in the *de novo* synthesis of those sites,[1–3] being associated with immortalization and the malignant phenotype of tumors.[4–7] The recent development of a highly sensitive polymerase chain reaction-based telomerase assay, called TRAP (telomeric repeat amplification protocol), has led to the detection of elevated telomerase activity in various cancer tissues of humans and rodents.[8–12]

Telomerase activity is reported to be depressed with cellular quiescence, contact inhibition, growth factor removal[13] and cell differentiation,[14] and reduced by factors such as chemotherapeutic agents in cultured human malignant cells.[15,16] As regards the *in vivo* situation, two reports appeared during the preparation of the present paper, showing that telomerase activity is decreased in human breast cancers[17] and in leukemia and solid tumors in children under chemotherapy.[18]

Osteosarcoma is the most prevalent and important malignant bone tumor of youth in man, having a poor prognosis with rapid growth and frequent distant metastasis, especially to the lung.[19] Previously, we established rat transplantable osteosarcomas with low and high metastatic potentials,[20–25] with increased expression of H-*ras*,[21] *nm23*,[21] c-*fos* and c-*jun*[22] and increased telomerase activity,[25] and we suggested that they might be useful as experimental tools to study the biological behavior of this type of malignancy. We also reported inhibitory effects of *cis*-diammine dichloroplatinum (CDDP), a frequently used agent for osteosarcomas treatment,[26–28] and O-(chloroace-

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tylcarbamoyl)fumagillol (AGM-1470), a well known anti-angiogenic agent[29, 30] considered to be a potent candidate for osteosarcoma treatment, using our osteosarcoma model.[31–33]

In this study, to determine the relation of telomerase activity to retardation of tumor growth during chemotherapy, a comparison of tumor volumes during and after administration of these two agents was made with rats bearing transplantable osteosarcomas.

MATERIALS AND METHODS

Animals Male Fischer 344 rats (Shizuoka Laboratory Animal Center, Shizuoka), 5 weeks old at the commencement, were used in the experiment. The animals were housed, four to a wire cage, in an air-conditioned room at 24°C, and given Oriental MF diet (Oriental Yeast Ind., Tokyo) and water *ad libitum*. The experiments were started when the recipient rats were 8 weeks old, weighing 180 g. Animals were killed under ether anesthesia in the morning on each of the sampling days. All animals were treated in accordance with our institutional guidelines for animal welfare.

Chemicals CDDP was obtained from Sigma Chemical Co. (St. Louis, MO). AGM-1470 was kindly supplied by Takeda Chemical Industries Co., Ltd., Osaka, and prepared for administration by dissolving in 100% ethanol for continuous administration.

Tumor and transplantation The transplantable osteosarcomas employed were derived from a spontaneous osteosarcoma, selected from lung metastatic lesions to obtain lines with high metastatic potential. Details regarding the methods of tumor production, handling and transplantations have been described previously.[20–25] All procedures were performed under aseptic conditions.

Experimental protocol Lung metastatic nodules, approximately 3 mm in diameter, were transplanted into the subcutaneous back space of rats with the following basic protocol. In the first experiment, groups 1–4 were given a single intravenous injection of CDDP at doses of 0, 0.625, 1.25 and 2.5 mg/kg body weight (b.w.), respectively, on day 14, and groups 5–8 received continuous administration of AGM-1470 through mini pumps, 2ML4 (ALZA, Palo Alto, CA), implanted in the subcutaneous space on the opposite side to the tumor transplantation site, at doses of 0, 2.5, 5 and 10 mg/kg b.w., respectively, from day 14 for 2 weeks. All animals were killed on day 28. Tumor sizes were measured and telomerase activity was analyzed by TRAP assay followed by densitometric quantification. In the second experiment, the recovery of tumor growth and telomerase activities was studied. Group 1′ received transplantation of osteosarcoma fragment tissue only; group 2′ received tumor transplantation followed by a single intravenous injection of 2.5 mg/kg b.w. CDDP on day 14; group 3′ received tumor transplantation followed by continuous administration of 10 mg/kg b.w. AGM-1470 by mini-pump from day 14 for 2 weeks. Subgroups of four rats were killed on days 7, 14, 21, 28, 35 and 42, and tumor size and telomerase activity were measured. Growth retardation was assessed using the formula: T/C = mean treated group tumor volume/non-treated group mean tumor volume.

Preparation of tissue extracts Tissue extracts were prepared by use of the procedures described previously,[5, 9] with some modifications.[10, 12] Briefly, frozen tissue was rinsed in cold buffer [23 m*M* N-2-hydroxyethylpiperazine-N′-3-propanesulfonic acid (HEPES; pH 7.5), 6.9 m*M* KCl, 2.3 m*M* $MgCl_2$, 2.3 m*M* dithiothreitol (DTT), 0.23 m*M* phenylmethanesulfonyl fluoride (PMSF), 2U/ml RNA guard (Pharmacia, Uppsala, Sweden), 2.3 m*M* leupeptin and 23 μM pepstain 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 12,000 rpm at 4°C. A one-fiftieth volume of 5 *M* NaCl was added and the samples were again centrifuged for 1 h at 100,000*g* at 4°C. The supernatants were collected and stored at −80°C until use. Protein concentration was determined by means of the DC protein assay (Bio Rad, CA). The average value was approximately 2 mg/ml.

Telomerase assay The telomerase assay was performed by a modification of the procedure described previously.[10, 12] Fifty microliters of a mixture containing polymerase chain reaction (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* EDTA, 0.05% NP40 and 0.05% Tween 20], 0.1 μg of TS primer (5′-AATCCGTCGAGCAGAGTT-3′), 50 μM dNTPs, 3 units of *Taq* DNA polymerase (Pharmacia), 0.4 μl of [α-^{32}P]dCTP (3000 Ci/mmol) and 5 μg of extract were incubated for 30 min at 25°C for extension of the TS primer by telomerase. Each reaction mixture contained 5×10^{-18} g (5 attograms) of internal telomerase assay standard (ITAS) for quantitative estimation of the levels of telomerase activity and identification of false-negative tumor samples containing *Taq* polymerase inhibitors.[34] ITAS is a 150-bp DNA standard, which is coamplified with telomerase activity products and is sufficiently long so that it does not interfere with the visualization of the telomerase ladder. Each mixture was heated to 90°C for 3 min and then 0.1 μg of the CX primer (5′-CCCT-TACCCTTACCCTTACCCTAA-3′) was added. Then the PCR procedure was performed in a thermal cycler 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 or CX primers were also included. Fifty microliters ali-

Table I. Dose-dependent Inhibitory Effects of CDDP or AGM-1470 on Telomerase Activity and Growth of Transplantable Osteosarcomas in Rats

Group	Chemical	Concentration (mg/kg)	No. of rats examined	Tumor volume[a,b] (mm^3)	Relative telomerase[a,c] activity (%)	T/C ratio[a,d]
1	CDDP	0	4	40057±1333	100	1.00
2	CDDP	0.625	4	40089±1731	98.2±2.5	1.00±0.04
3	CDDP	1.25	4	16194±2675[e]	76.0±2.9[e]	0.40±0.07
4	CDDP	2.5	4	4236±1090[e]	18.2±2.2[e]	0.08±0.06
5	AGM-1470	0	4	42509±5352	100	1.00
6	AGM-1470	2.5	4	36437±5398	95.7±1.7	0.86±0.13
7	AGM-1470	5	4	12482±2000[e]	66.2±2.9[e]	0.24±0.15
8	AGM-1470	10	4	5159±1442[e]	24.5±1.7[e]	0.14±0.05

a) Data are mean±SD values.
b) Tumor volume was calculated as follows; tumor volume (mm^3)=$0.5\times a\times b^2$ (*a* and *b* are the longest and the shortest diameters).
c) Telomerase activities for groups 2 to 4 are relative to group 1 and those for groups 6 to 8 are relative to group 5.
d) Mean tumor volumes for groups 2 to 4 are relative to group 1 and those for groups 6 to 8 are relative to group 5.
e) Significantly different from group 1 or 5 ($P<0.001$).

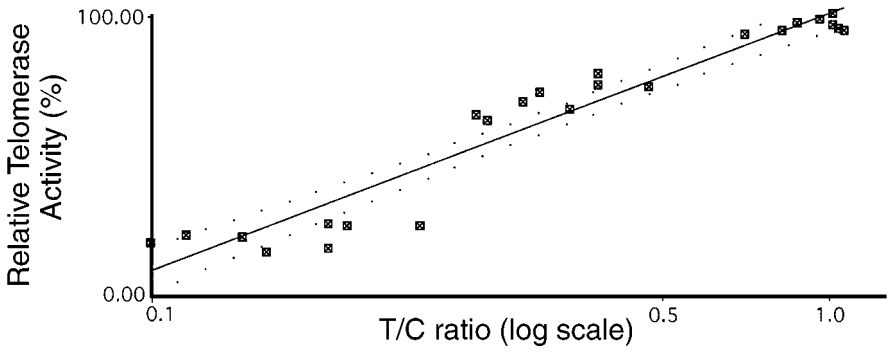

Fig. 1. Regression curve for the relationship between relative telomerase activity and T/C ratio for transplantable osteosarcomas in rats treated with CDDP at doses of 0–2.5 mg/kg b.w. or AGM-1470 at doses of 0–10 mg/kg b.w. on day 14. The curve was prepared with the values shown in Table I. $r=0.96$ ($P<0.0001$), $Y=(80.04)\log X+101.42$.

Table II. Increased Telomerase Activities and Tumor Re-growth as a Function of Time after Tumor Transplantation in Rats Treated with CDDP or AGM-1470[a]

Days after transplantation	No. of rats examined			Tumor volume (mm^3)[b,c]			Relative telomerase activity (%)[b,d]			T/C ratio[b,e]	
	G1′	G2′	G3′	G1′	G2′	G3′	G1′	G2′	G3′	G2′	G3′
7	4	4	4	66.2±23	65.0±15	56.4±12	100	NE	NE	NE	NE
14	4	4	4	4528±1967	5685±624	4770±948	99.3±5.8	99.7±4.7	103±3.1	1.25±0.14	1.05±0.21
21	4	4	4	16906±5000	4524±2615[f]	4976±2350[f]	113.8±5.0	23.5±2.3[f]	20.5±3.7[f]	0.27±0.16	0.29±0.14
28	4	4	4	40298±13315	4215±2213[f]	5090±1698[f]	111.6±7.5	22.2±4.0[f]	52.5±3.5[f]	0.10±0.05	0.12±0.04
35	4	4	4	48711±17132	3306±1591[f]	14499±2061[f]	111.7±3.5	18.2±8.2[f]	61.0±4.5[f]	0.07±0.03	0.30±0.04
42	4	4	4	50095±11431	9987±6154[f]	28543±3262[f]	108.7±2.7	46.5±3.1[f]	92.5±3.7[f]	0.20±0.12	0.57±0.07

a) Experimental G1′ (group 1′) received tumor transplantation only, G2′ (group 2′) received tumor transplantation followed by CDDP on day 14, G3′ (group 3′) received tumor transplantation followed by AGM-1470 from day 14 for 2 weeks.
b) Each value represents a mean±SD.
c) Tumor volume was calculated as follows; tumor volume (mm^3)=$0.5\times a\times b^2$ (*a* and *b* are the longest and the shortest diameters).
d) Telomerase activities in groups 2′ and 3′ are relative to group 1 on day 7.
e) Mean tumor volumes in groups 2′ and 3′are relative to group 1′ at each day when animals were killed after transplantation.
f) Significantly different from group 1′ ($P<0.01$).
NE: not examined.

quots of PCR products were electrophoresed on 12% non-denaturing polyacrylamide gels. To evaluate the relative level of telomerase activity in each sample, the polyacrylamide gels were exposed to a Phospho Imaging Plate (Fujix, Tokyo), and the intensity of the TRAP ladder was compared to that of the ITAS signal as described previously,[34] using a Bio-Imaging Analyzer (BAS1000; Fujix) and MacBAS software (Fujix). Relative telomerase activities were quantified by taking the ratio of the entire TRAP ladder to the signal of amplified ITAS.

To assess the direct effect of CDDP and AGM-1470 on telomerase activity, *in vitro* analysis was performed by adding the drugs directly to the telomerase reaction mixture, including protein extracted from transplantable osteosarcomas, before primer addition. Samples were incubated for 1 h and processed for the TRAP assay.

Statistical analyses Statistical analyses were performed using a personal computer and InStat graphPAD software (San Diego, CA) as described previously.[35] To assess the statistical significance of inter-group differences in quantitative data, Dunnett's multiple comparison test was performed after one-way analysis of variance to determine variation among the group means followed by Bartlett's test to determine the homogeneity of variance. The correlation between telomerase activity and T/C rate was evaluated using Spearman's correlation and linear regression functions. Significant differences from zero of the slope of each regression function were assessed using the ANOVA table. Lack of significant departure from linearity for each regression function was confirmed doubly by Runs and ANOVA tests.

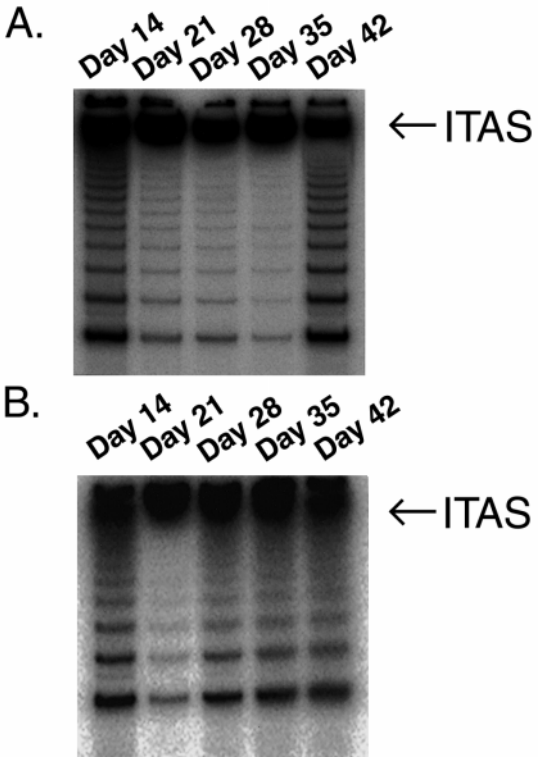

Fig. 2. Representative telomere patterns of transplantable osteosarcomas in rats treated with CDDP at a dose of 2.5 mg/kg b.w. (A) or AGM-1470 at a dose of 10 mg/kg b.w (B).

RESULTS

Dose-dependence of the effects of CDDP and AGM-1470 on telomerase activity and volumes of transplantable osteosarcomas in rats on day 28 In groups 3, 4 , 7 and 8 in the first experiment, both relative telomerase activity and tumor volumes were significantly, dose-

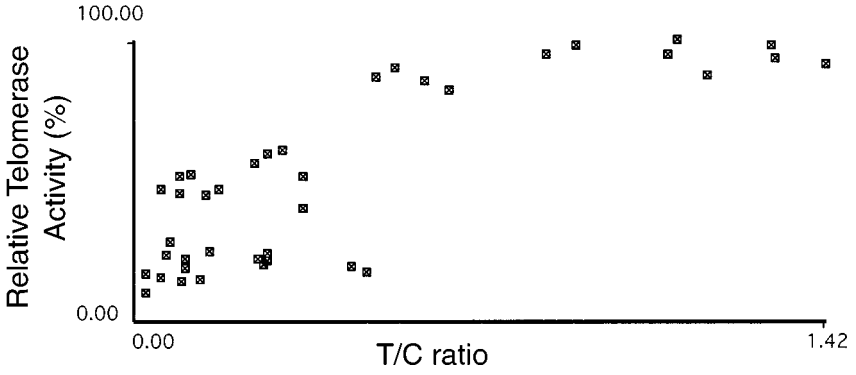

Fig. 3. The relationship between relative telomerase activity and T/C ratio for transplantable osteosarcomas in rats treated with CDDP at a dose of 2.5 mg/kg b.w. on day 14 or AGM-1470 at a dose of 10 mg/kg b.w. starting from day 14 for 2 weeks. The dots were prepared with the values shown in Table II. $r = 0.73$ ($P<0.0001$).

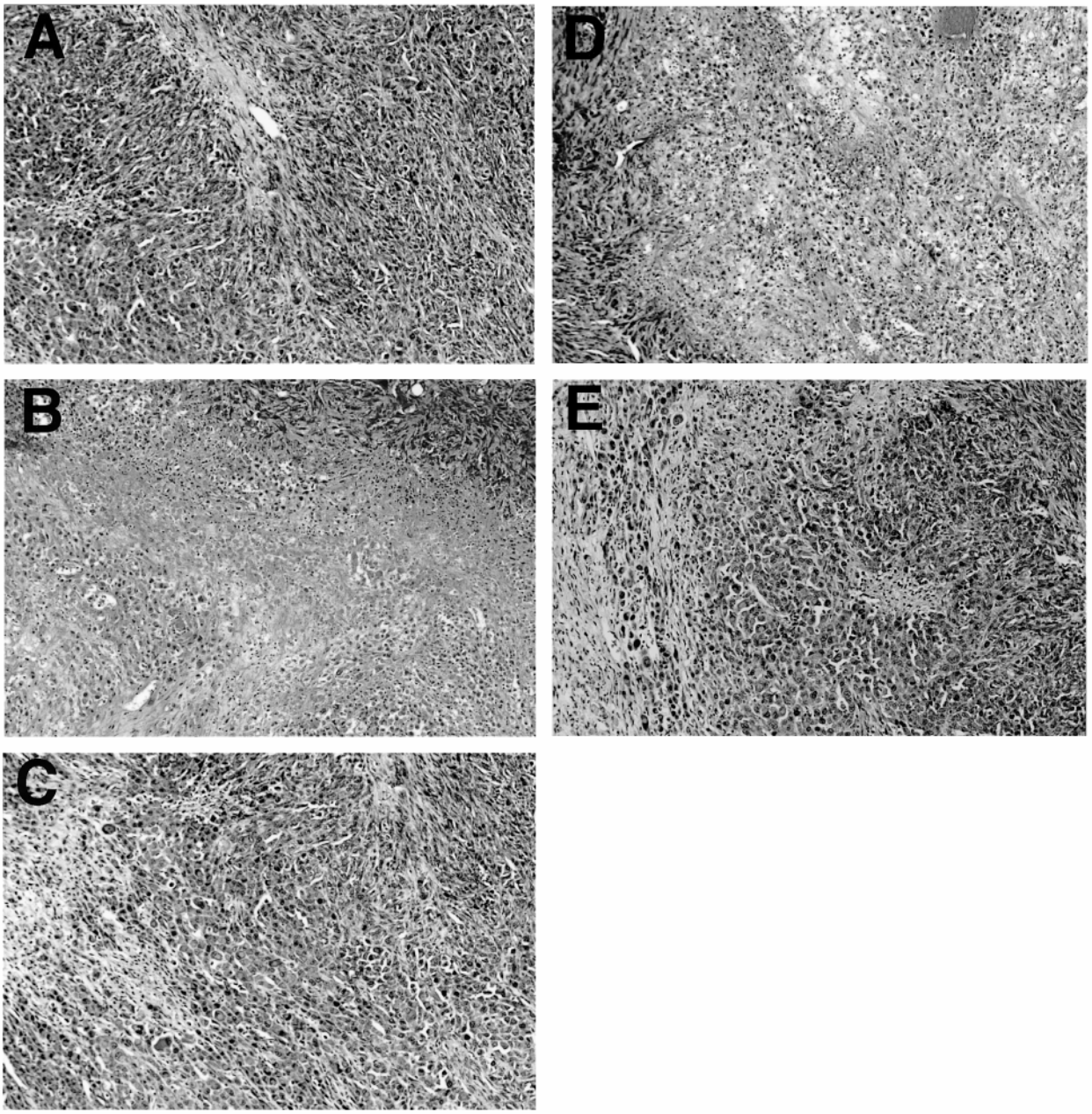

Fig. 4. Histology of the transplanted tumors: A, group 1′ on day 7, without treatment; B, group 2′ on day 21, treated with CDDP at 2.5 mg/kg b.w., showing wide necrotic area; C, group 2′ on day 42, treated with CDDP at 2.5 mg/kg b.w., showing viable tumor cells; D, group 3′ on day 21, treated with AGM-1470 at 10 mg/kg b.w., showing necrosis; E, group 3′ on day 42, treated with AGM-1470 at 10 mg/kg b.w., displaying viable tumor cells. (H-E stain, ×200)

dependently decreased as compared to controls, the most effective doses being 2.5 mg/kg b.w. for CDDP and 10 mg/kg b.w. for AGM-1470 (Table I). In Fig. 1, the regression functions are graphed; the relative telomerase activity was proportional to the logarithm of the T/C ratio ($r = 0.96$, $P < 0.0001$).

Time-course of the effects of CDDP and AGM-1470 on telomerase activity and volumes of transplantable osteosarcomas in rats on days 14–42 In the second experiment, tumor volumes in groups 2′ and 3′ were significantly smaller than the group 1′ values on days 21, 28, 35 and 42 (Table II). Representative telomere patterns

with ITAS are shown in Fig. 2 (Fig. 2A, group 2′; Fig. 2 B, group 3′). Telomerase activity decreased to 18.2% on day 35 in group 2′ and 20.5% on day 21 in group 3′. However, increases to 46.5% in group 2′ and to 92.5% in group 3′ on day 42 were observed in line with the re-growth of tumors. As shown in Fig. 3, a statistically significant correlation between relative telomerase activity and the T/C ratio was obtained ($r=0.73$, $P<0.0001$). The histology of transplantable osteosarcomas without treatment or treated with CDDP and AGM-1470 on days 21 and 42 is shown in Fig. 4. During the effective periods for CDDP or AGM-1470, predominant necrosis were seen in tumors when telomerase activities were reduced (Fig. 4, B and D), but, once the agents became ineffective, viable cells appeared and telomerase activities increased (Fig. 4, C and E). Therefore, telomerase activity well reflected the therapeutic effect in transplantable osteosarcomas in rats. *In vitro* direct effects of CDDP and AGM-1470 on telomerase activity were not observed (data not shown).

DISCUSSION

The present experiment demonstrated changes in telomerase activities and osteosarcoma growth in rats during and after treatment with CDDP or AGM-1470, a statistically significant correlation between telomerase activity and the T/C ratio being obtained.

Recently, a number of cytotoxic agents have been reported to cause a decline of telomerase activity in cultured cells. Zhu *et al.*[15] described a marked reduction of telomerase activity in SW480 colon carcinoma cells treated with doxorubicin, 5-fluorouracil and methotrexate, and suggested that this might have been due to blockage of cell progression through the cell cycle. Faraoni *et al.*[16] reported a decline of telomerase activity in T-cell leukemia Jurkat cells, histiocytic U937 cells and breast adenocarcinoma MCF-7 cells treated with doxorubicin, temozolomide and CDDP, demonstrating that the decrease of telomerase activity paralleled cell growth impairment. They also suggested that detectable telomerase activity remaining after treatment with antineoplastic agents most likely reflected the activity of the remaining viable cells. Likewise, telomerase activity is decreased during chemotherapy of human breast cancers[17] and pediatric malignancies.[18]

The present results have experimentally confirmed that telomerase activity reflects chemotherapeutic effect. Our first experiment shows that the efficacy of chemotherapeutic agents is linked to decreased telomerase activity. The fact that detectable telomerase activity remained, suggesting the continued existence of viable tumor cells, was in line with the recovery observed in the second experiment. Thus, after the effective periods of CDDP and AGM-1470 therapy, increase in the telomerase activity correlated with tumor re-growth. While both agents were effective, there are differences in their antitumor mechanisms. CDDP is a potent DNA-damaging agent, whereas AGM-1470 is not. AGM-1470 affects tumor cells indirectly by inhibition of new vessel formation.[29, 30] Histologically, the vessels in tumors treated with AGM-1470 at effective doses are sparse,[31] but this is not so in CDDP-treated tumors. However, wide necrotic areas were observed in tumors treated with both agents, accompanied with decreased telomerase activities. Since the present *in vitro* results indicate that CDDP and AGM-1470 do not directly affect telomerase activity, our *in vivo* findings suggest that the correlation between decreased telomerase activity and tumor growth retardation might be a reflection of impaired cell growth rather than its cause.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid (to YK and to YMiy) from the Ministry of Education, Science, Sports and Culture and by a Grant-in-Aid (to YK) from the Ministry of Health and Welfare for the 2nd Term Comprehensive 10-Year Strategy for Cancer Control, Japan.

(Received June 17, 1998/Revised July 21, 1998/Accepted August 3, 1998)

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