
Kinetic Analysis of Malignant Fibrous Histiocytoma Cells Treated with Anticancer Agents In Vivo

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To clarify the in vivo effects and the changes in kinetic parameters of soft tissue sarcoma caused by anticancer agents, we performed double-labeling using bromo- (BrdU) and iododeoxyuridine (IUdR). A malignant fibrous histiocytoma cell line was transplanted into nude mice, and treated with 3 anticancer agents: cisplatin, doxorubicin and vincristine. The following parameters were determined: BrdU labeling index (LI), duration of S-phase (Ts), total cell cycle time (Tc), Ki-67 labeling index (KLI), PCNA labeling index (PCNA-LI), and actual tumor volume doubling time (Tv). In addition, the apoptotic cell ratio (apoptotic index; AI) was calculated in the serial specimens using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL).

Vincristine inhibited the tumor growth the most effectively (Tv: -5.8 days). Doxorubicin suppressed the tumor growth better (Tv: 12.7 days) than cisplatin (Tv: 9.1 days). Tc values showed significant differences among the drug groups (ANOVA analysis; p<0.05), and also correlated with Tv (Pearson’s analysis; R>0.7, p<0.05) on Day 1. AI peaked on Day 10 and decreased afterward in every drug group.

Anticancer agents effectively suppress tumor growth not only by necrosis and apoptosis, but also by prolonging Tc. Tc values are possibly useful for planning chemotherapy for sarcoma patients.

Key words: Cell kinetics, Bromodeoxyuridine (BrdU), Iododeoxyuridine (IUdR), Soft tissue sarcoma, Apoptosis

I. Introduction

The clinical effects of anticancer agents on bone and soft tissue sarcomas are uncertain, particularly in the case of the latter. In addition, the results of in vitro chemosensitivity tests sometimes differ from the clinical results [18]. On the other hand, the results of in vivo tests, such as the protocol of Battelle Columbus Laboratories, are comparable to clinical findings. However, this protocol is labor intensive [16, 17].

The relationship among various proliferating tracers for clinical prognosis has been examined in bone and soft tissue tumors by labeling the tracers with BrdU [5, 7, 8], Ki-67 [24], and PCNA [2-4]. Monoclonal antibodies, specifically thymidine analogues such as BrdU and IUdR, have opened up new areas of research because they can be incorporated into DNA at the S-phase of the cells. This double-labeling method facilitates quantifying the proliferative potential of individual tumors. This method can be used to determine the BrdU-labeling index (LI), duration of S-phase (Ts) and total cell cycle time (Tc). These cell cycle parameters can also be calculated while the histologic architecture of the tumor specimen is preserved.

We have already reported that potential doubling time can be a prognostic factor in patients with bone and soft tissue sarcomas [15].

The objective of this study was to examine the cell kinetics of soft tissue sarcoma cells in nude mice treated with various anticancer agents by analyzing the above-mentioned parameters in vivo. The correlations between tumor suppression and various growth parameters were statistically analyzed. Furthermore, apoptotic cell ratio (apoptotic index; AI) was calculated and compared with these parameters.

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Fig. 1. A: Photomicrograph showing malignant fibrous histiocytoma cells labeled by immunohistochemical double-staining of BrdU and IUdR. Both IUdR- and BrdU-positive nuclei were stained black, IUdR-positive nuclei blue, and BrdU-positive nuclei brown (one day after treatment with vincristine, original magnification $\times 100$). B: Photomicrographs showing apoptotic cells stained with terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling. Apoptotic nuclei and bodies were stained brown (9 days after the treatment of vincristine, original magnification $\times 100$).
II. Materials and Methods

Tumor cells of a soft-part malignant fibrous histiocytoma (MFH) excised from the left thigh of a 51-year-old male patient had been maintained in nude mice in our institute. The MFH cells of the 12th passage, which were considered to have originated from a clonal cell, were transplanted subcutaneously into the backs of 60 nude mice to produce 120 individual tumors (2 tumor nodules in each mouse). The tumors in the mice were randomized into control and drug groups (30 tumor nodules/group). When the tumor volume reached 100 mm$^3$ (approximately 2 weeks after the subcutaneous transplantation), the first dose of each drug was administered. Three drugs that are frequently used clinically were selected for this study: cisplatin (CDDP), doxorubicin (ADM) and vincristine (VCR). An equitoxic dose of $1/3$ LD50 of each drug was given intraperitoneally 3 times every 4 days (Days 0, 4 and 8). LD50 concentrations of the drugs were 14.6 mg/kg (CDDP), 14.4 mg/kg (ADM), and 4.3 mg/kg (VCR), respectively. Physiologic saline solution was administered into the control mice. Three mice (6 nodules) from each group also received intraperitoneal injections of IUdR (80 mg/kg) and BrdU (80 mg/kg) with 2-hr intervals on Days 1, 5, 9, 17 and 33 after the first treatment. They were then sacrificed 1 hr after the double-labeling procedure.

The specimens were prepared and stained with BrdU and IUdR. BrdU was stained brown with diaminobenzidine (DAB) by the avidine-biotin-peroxidase complex (ABC) procedure, and IUdR was stained blue with fast blue dye by the alkaline phosphatase-anti-alkaline phosphatase (APAAP) complex procedure (Fig. 1A). Since this double-labeling procedure has a time lag of 2 hr, the proportion (dS) of cells not in the DNA synthesis phase during the double-labeling experiments could be derived from a stain sample. The values of Ts and Tc were therefore calculated on the basis of the dS [15, 19, 20, 22]. In brief, they were calculated as follows:

\[
\begin{align*}
\text{dS} &= \frac{\text{blue}}{\text{brown} + \text{double}} \\
\text{Ts} &= \frac{\text{time lag}}{\text{dS}} \\
\text{Tc} &= \frac{\text{Ts}}{\text{LI} \times \text{growth fraction}}
\end{align*}
\]

The growth fraction was considered to be an approximate value of the PCNA labeling index (PCNA-LI), and it was obtained as follows. The serial sections were immunohistochemically stained with Ki-67 and PCNA. The Ki-67 labeling index (KLI), and the PCNA-LI were also calculated. Immunohistochemical staining of Ki-67 was carried out after the autoclaving procedure at 121°C for 10 min in 10 mM citrate buffer containing 0.1% NP40. Antibodies for IUdR (IU-4; dilution 1:100, Caltag Lab., Inc.), BrdU (Br-3; dilution 1:750, Caltag Lab., Inc.), Ki-67 (EPOS-Ki-67; dilution 1:1, DAKO Co.), and PCNA (PC10; dilution 1:100, Novocastra Lab., Inc.) were utilized in this study.

For in situ visualization of apoptotic cells, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) was performed [6, 9, 11]. The TUNEL technique was performed using an apoptosis detection kit (ApopTag Plus In Situ Apoptosis Detection Kit, Oncor, Gaithersburg, MD). The serial sections that were treated with proteinase K were used
for evaluating the apoptotic cells. The reaction time was 5 min for proteinase K (ApopTag Plus In Situ Apoptosis Detection Kit, Oncor, Gaithersburg, MD) and 100 min for both TdT and digoxygenin-dUTP. Some sections were pre-treated with DNase I (Sigma Chemical Company, Japan) as a positive control for this method. The AI was expressed as the ratio of positively-stained tumor cells and bodies to all tumor cells (Fig. 1B).

In addition, the actual tumor volume was measured twice a week to determine the tumor growth curve for each drug group, and the actual tumor volume doubling time (Tv) was calculated from the curve on a semi-logarithmic graph (Fig. 2). This protocol was similar to the method used by the Battelle Columbus Lab [16, 17], in which tumor volume was calculated as follows:

\[ \log V = \log 2V_0 \times \text{days} \times 1/Tv \]

1/Tv: the reciprocal of Tv, V: tumor volume

Vo: surviving transplanted tumor volume

In brief, the more effective the anticancer agent, the lower the value of 1/Tv became. We therefore analyzed the linear correlation between 1/Tv and the other cell kinetic parameters.

The linear correlation between 1/Tv and the kinetic parameters was statistically examined using Pearson’s correlation coefficient (Pearson’s analysis; Stat View 4.11J: Abacus Concepts, Inc., Berkeley, CA ). The differences in values between control and drug groups were analyzed using one-way factorial ANOVA and multiple comparison tests (ANOVA analysis; Stat View 4.11J).

### III. Results

The tumors from the control group had strong proliferative activity (Tv: 5.2 days). VCR inhibited tumor growth the most effectively (Tv: -5.8 days). Antitumor effects were also observed with ADM (Tv: 12.7 days), followed by CDDP (Tv: 9.1 days), which was the least effective (Fig. 2). The values of ADD on Days 17 and 33 could not be obtained due to experimental animal deaths caused by a side effect of the drug. For this reason, statistical examination on these days was performed without the values in the ADM group.

The changes in the values of LI, KLI, and PCNA-LI...
occurred in a time-dependent manner (Fig. 3). Significant differences in the values of each group were observed during Day 9 (ANOVA analysis; p<0.05). The values of each drug on Day 1, however, showed no significant difference. The correlation between LI and 1/Tv was significant on Days 5 and 9. A correlation was noted on Days 9 and 17 for the KLI value, and on Days 5, 9 and 17 for the PCNA-LI. The relationship with 1/Tv was not significant on Day 1 (Table 1).

VCR showed a prolonged Ts value (34.0 hr) on Day 1. The Ts of each drug group also tended to be prolonged on Day 1. The Ts on Days 1 and 33 also correlated with 1/Tv (Table 1). VCR also showed a prolonged Tc value (112.4 hr) on Day 1, and the value for each drug group was significantly different (ANOVA analysis; p < 0.05). The prolongation of Tc with VCR continued even after completion of the treatment (corresponding to Day 8). In the ADM- and CDDP-groups, the prolongation of Tc was due to an increase in the dose, but each Tc value rapidly returned to the baseline after Day 8 (Fig. 4A). The difference in Tc value for each group was significant throughout the experimental period (ANOVA analysis; p<0.05). Interestingly, Tc on Day 1 showed a strong correlation with 1/Tv (Pearson’s analysis; Tc = 73.956 - 221.884 /Tv; R^2 = 0.713, p<0.0001, Fig. 4B).

All the drugs used in this study induced apoptosis, whereas apoptosis was almost never detected in the control group (less than 1%). The AI depended on both the time and doses of drugs administered, and each AI value was significant among the drug groups (Fig. 5). However, the correlation between AI and 1/Tv was not significant in the early stage.

IV. Discussion

The clinical use of anticancer agents in the treatment of soft tissue sarcoma is controversial. It is also a problem that the results of in vitro chemosensitivity tests on malignant tumors do not necessarily agree with in vivo drug effects [18]. It seems especially difficult to determine the actual chemosensitivity of sarcoma cells using cell sus-
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pensions prepared by destroying tissue structure because of the histologic heterogeneity of soft tissue sarcomas. Even now, the "classic" in vivo drug sensitivity test reported by Battelle Columbus Laboratories seems to be the most accurate [17]. However, this procedure is not practical for clinical use because it is labor intensive. In the present study, this method was carried out and the actual tumor volume of each drug group was measured in order to compare it with other cell kinetic parameters. As previously reported, tumor volume was useful in determining the in vivo efficacy of each anticancer agent. However, 7 weeks were required to confirm the efficacy of the drugs.

It is important to understand how soft tissue sarcoma cells respond to treatment with various anticancer agents. The present study demonstrated that apoptosis was induced by these drugs in vivo. Apoptosis is a mode of cell death characterized by distinctive biochemical and morphological features [1]. The evaluation of apoptosis is an important aspect of the study of chemical carcinogenesis [9]. Apoptosis is related to angiogenesis in tumor progression [11]. There has also been increasing attention directed to the hypothesis that apoptosis plays a role in the response to cancer treatment, including chemotherapy [12]. Radiation exposure, anticancer agents and hyperthermia were also reported to induce necrosis and apoptosis of sarcoma cells in vitro [23, 26]. On the other hand, a few studies on the apoptosis of tumor cells in vivo have been reported before [9, 11-13]. However, almost all of them were in vivo studies of carcinoma cells. The relationship between apoptosis and kinetic parameters of sarcoma cells has never been described, to the best of our knowledge. In the present study, the AIs in the middle and late stage correlated well with reduction in tumor volume. This finding reflected the efficacy of the drugs administered, but the degree of apoptosis in the early stage did not predict drug efficacy.

Our results showed that VCR had the strongest activity for tumor suppression against the cell line of soft-part malignant fibrous histiocytoma. Combination multi-drug chemotherapy including VCR has been reported to be clinically effective in the treatment of malignant fibrous histiocytoma and other soft tissue sarcomas [10, 19]. In the present study, VCR markedly prolonged the Tc value (total cell cycle time), compared with the other agents. It was demonstrated that the more effective the drug was, the greater and more sensitive the Tc value became in the early stages. Furthermore, the Tc values on Day 1 correlated well with the tumor growth indicated by 1/Tv. These findings showed that cell damage caused by the drugs prolonged the cell cycle time both in the early and late stages. Unlike these findings, apoptosis was induced in the early stage (around the Day 10) but not in the late stage of the present experiment. We speculate that some of the sarcoma cells with prolonged cell cycle time became apoptotic in the early stage. The other cells remained viable, but with prolonged cell cycle time even in the late stage.

As for the application of the present results to clinical treatment, we have already reported that the potential doubling time of bone and soft tissue sarcomas in nude mice is a useful prognostic indicator, despite differences in histologic diagnoses [15]. It should be noted that neither the Tc nor Ts values reflect the over all status of the tumor cells. They do not directly reflect the Tv, since only the surviving tumor cells in the cell cycle can be assessed using these parameters. Apoptotic cells (non viable cells) should also be evaluated by the TUNEL method to analyze the tumor precisely. However, cell kinetic changes in the early stage can be evaluated using Tc values. Tc values can be calculated without waiting several weeks for tumor growth in nude mice. It is possible that the chemosensitivity of each drug can be determined by measuring Tc values in the early stage.

Although more data are necessary for forming definite conclusions, this study demonstrated a possible application of Tc values for practical use in in vivo chemosensitivity testing. If these thymidine analogues (BrdU and IUDR) could be injected into sarcoma patients, as has been done in other cancer patients [14, 20-22, 25, 27], Tc values could be obtained directly from the surgical specimens. The serial measurement of this parameter before (via biopsy) and after chemotherapy (via definitive surgical specimen) may become useful in determining the chemosensitivity much earlier. As far as the accuracy of the parameters is concerned, in situ examination with direct drug administration to humans would be preferable to animal studies. However, BrdU and IUDR are used as sensitizers in radiation therapy. Therefore, administration of these nucleic acid analogues in humans is an experimental procedure limited to institutions at present. Instead of testing on human patients, sarcoma tissue transplanted into nude mice can be used for this purpose. According to our findings, Tc values of greater than 75 hr corresponded to negative Tv values. When Tc values become greater than 75 hr after the administration of an anticancer agent, the drug can be considered to be effective in suppressing tumor growth.

In conclusion, anticancer agents are effective in suppressing the growth of sarcoma cells not only by inducing apoptosis, but also by extending the total cell cycle time (Tc). Calculated Tc values on Day 1 are as useful as Tv values in determining drug efficacy. In addition, chemosensitivity can be evaluated much earlier using Tc values rather than Tv values. Tc values are useful in planning therapeutic strategies for sarcoma patients.

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VI. References

1. Ansari, B., Coates, P. J., Greenstein, B. D. and Hall, P. A.: In
situ end-labelling detects DNA strand breaks in apoptosis and other physiological and pathological states. J. Pathol. 170; 1–8, 1993.


