Feasibility of ^{99m}Tc-Annexin V for Repetitive Detection of Apoptotic Tumor Response to Chemotherapy: An Experimental Study Using a Rat Tumor Model

Yuji Kuge, PhD[1]; Masayuki Sato, BS[1–3]; Songji Zhao, MD[2]; Toshiki Takei, MS[2]; Kunihiro Nakada, MD[2]; Koh-ich Seki, PhD[1,3]; H. William Strauss, MD[4]; Francis G. Blankenberg, MD[5]; Jonathan F. Tait, PhD[6]; and Nagara Tamaki, MD[2]

[1]Department of Tracer Kinetics, Graduate School of Medicine, Hokkaido University, Sapporo, Japan; [2]Department of Nuclear Medicine, Graduate School of Medicine, Hokkaido University, Sapporo, Japan; [3]Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Japan; [4]Department of Nuclear Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York; [5]Pediatric Radiology, Stanford University School of Medicine, Palo Alto, California; and [6]Department of Laboratory Medicine, University of Washington, Seattle, Washington

Annexin V (annexin A5), a human protein with a high affinity for phosphatidylserine, labeled with ^{99m}Tc can detect apoptosis in vivo. In the repetitive detection of apoptosis with ^{99m}Tc-annexin V, however, the specific binding of annexin V to phosphatidylserine might affect the subsequent detection of apoptosis with this compound. To determine whether there is interference with repetitive doses of annexin V, we evaluated the effects of previous administration of cold annexin V on accumulation of ^{99m}Tc-annexin V in tumors in an experimental tumor model. **Methods:** Rats bearing hepatoma received cyclophosphamide (150 mg/kg, intraperitoneally) 11 d after the tumor inoculation. Cold annexin V (20 μg/kg, intravenously) was administered 24 h before or after the cyclophosphamide treatment ($n = 7$/group). ^{99m}Tc-Annexin V was injected intravenously (radioactive dose, 5–23 MBq/kg; mass dose, 20 μg/kg), and radioactivity in tissues was determined 6 h later. **Results:** Accumulation of ^{99m}Tc-annexin V in tumors was not significantly affected by previous treatment with cold annexin V before or after chemotherapy. **Conclusion:** These results demonstrate the feasibility of ^{99m}Tc-annexin V imaging for repetitive detection of apoptosis, which is highly required in the clinical setting.

Key Words: ^{99m}Tc-annexin V; apoptosis; tumor; chemotherapy; rat

J Nucl Med 2004; 45:309–312

Apoptosis plays an important role in both normal physiology and many disease processes (*1*). One of the earliest events in apoptosis is the externalization of phosphatidylserine, a membrane phospholipid normally restricted to the inner leaflet of the lipid bilayer. Annexin V (annexin A5), a human protein with a high affinity for membrane-bound phosphatidylserine (*2*), can be labeled with fluorescent markers for in vitro detection of apoptotic cells (*3*) and with radioactive agents, such as ^{99m}Tc, to detect apoptosis in vivo (*4*).

Successful chemotherapy or radiotherapy of tumors induces apoptosis in neoplastic cells (*5*). Previous studies indicated that radiolabeled annexin V imaging can detect this apoptotic tumor response in vivo in experimental models (*4,6,7*) and in patients (*8*). In a clinical setting, annexin V injection and imaging have been performed both immediately before and after an initial treatment. The pre- and posttreatment injections may be only a few days apart. Because annexin V binds to about 3 phosphatidylserine molecules on the cell surface with a nanomolar affinity, it is possible that the first injection of annexin V may still be resident on the cell surface, tying up phosphatidylserine and thereby compromising the ability of the second dose to localize. To determine whether there is interference with repetitive doses of annexin V, we evaluated the effects of previous administration of cold annexin V on accumulation of radiolabeled annexin V in tumors in an experimental tumor model.

MATERIALS AND METHODS

All procedures involving animals were performed in accordance with the institutional guidelines of Hokkaido University and the current laws in Japan.

Male Wistar King Aptekman/Hok rats (supplied by the Experimental Animal Institute, Graduate School of Medicine, Hokkaido University) were inoculated with a suspension of KDH-8 rat hepatoma cells (1×10^6 cells per rat) into the left calf muscle and

Received Jul. 16, 2003; revision accepted Oct. 23, 2003.

For correspondence or reprints contact: Nagara Tamaki, MD, Department of Nuclear Medicine, Graduate School of Medicine, Hokkaido University, Kita15 Nishi7, Kita-ku, Sapporo 060-8638, Japan.

E-mail: natamaki@med.hokudai.ac.jp

TABLE 1
Treatment of Each Group of Rats Inoculated with Hepatoma Cells

Group	Days after tumor inoculation			
	Day 10	Day 11	Day 12	Day 13
Group A (n = 7)	Cold annexin V	CP	^{99m}Tc-annexin V	
Group B (n = 7)	NT	CP	^{99m}Tc-annexin V	
Group C (n = 6)	NT	NT	^{99m}Tc-annexin V	
Group D (n = 7)	NT	CP	Cold annexin V	^{99m}Tc-annexin V
Group E (n = 7)	NT	CP	NT	^{99m}Tc-annexin V
Group F (n = 6)	NT	NT	NT	^{99m}Tc-annexin V

Cold annexin V = 20 μg/kg, intravenously; CP = cyclophosphamide, 150 mg/kg, intraperitoneally; ^{99m}Tc-annexin V = a radioactive dose of 5–23 MBq/kg, a mass dose of 20 μg/kg, intravenously; NT = not treated.

divided into 6 groups (n = 6–7/group; Table 1). Rats in groups A, B, D, and E received a single dose of cyclophosphamide (150 mg/kg, intraperitoneally) 11 d after tumor inoculation (day 11). Rats in group A received unlabeled (cold) recombinant human annexin V (annexin A5; Alexis Corp.; 20 μg/kg, intravenously) 24 h before the cyclophosphamide treatment (day 10), and rats in group C received cold annexin V (20 μg/kg, intravenously) 24 h after the cyclophosphamide treatment (day 12) under light ether anesthesia. Rats in groups B and D served as controls for groups A and C, respectively, and rats in groups C and F served as untreated controls.

Annexin V-117, a mutant molecule of annexin V engineered to contain a binding site for ^{99m}Tc without reducing the affinity for phosphatidylserine, was produced by expression in *Escherichia coli*. The protein was labeled with ^{99m}Tc to produce ^{99m}Tc-annexin V as previously described (*9*). The specific activity of ^{99m}Tc-annexin V was 0.25–1.15 MBq/μg. With the tumor-bearing rats (body weight, 170–230 g) under light anesthesia, ^{99m}Tc-annexin V (radioactive dose, 5–23 MBq/kg; mass dose, 20 μg/kg) was injected intravenously. Groups A–C were injected on day 12, and groups C–E on day 13. Six hours after ^{99m}Tc-annexin V injection, the animals were sacrificed and the tumors, blood, and samples of normal tissues were collected. The tissue samples were weighed, and radioactivity was determined with a well-type scintillation counter (1480 Wizard 3″; Wallac Co., Ltd.). The accumulation of ^{99m}Tc-annexin V in the tissues was expressed as percentage injected dose per gram of tissue after normalization to the animal's weight ((%ID/g) × kg).

All values are shown as mean ± SD. Statistical analysis was performed using the unpaired Student t test to evaluate the significance of differences in values between the 2 groups. A 2-tailed value of $P < 0.05$ was considered significant.

RESULTS

Tissue distribution of ^{99m}Tc-annexin V is shown in Table 2. In untreated rats (groups C and F), uptake of ^{99m}Tc-annexin V was highest in the kidneys, followed in decreasing order by the spleen, liver, and bone marrow. Cyclophosphamide treatment (groups B and E) significantly increased the accumulation of ^{99m}Tc-annexin V in several tissues, including the tumor, thymus, spleen, and bone marrow.

Cold annexin V injection before cyclophosphamide treatment did not significantly affect the accumulation of ^{99m}Tc-annexin V in tumors (group A, 0.021 ± 0.002 [%ID/g] ×

TABLE 2
Biodistribution of ^{99m}Tc-Annexin V in Rats Inoculated with Hepatoma Cells

Tissue	Group A	Group B	Group C	P*	P†	Group D	Group E	Group F	P‡	P§
Blood	0.013 ± 0.001	0.013 ± 0.001	0.011 ± 0.001	NS	<0.01	0.011 ± 0.001	0.011 ± 0.001	0.009 ± 0.001	NS	<0.01
Tumor	0.021 ± 0.002	0.022 ± 0.003	0.017 ± 0.002	NS	<0.05	0.026 ± 0.002	0.026 ± 0.002	0.017 ± 0.003	NS	<0.01
Muscle	0.002 ± 0.001	0.003 ± 0.001	0.002 ± 0.001	NS	NS	0.003 ± 0.001	0.003 ± 0.001	0.002 ± 0.001	NS	<0.01
Thymus	0.027 ± 0.008	0.023 ± 0.004	0.005 ± 0.001	NS	<0.01	0.012 ± 0.003	0.011 ± 0.002	0.004 ± 0.001	NS	<0.01
Spleen	0.489 ± 0.064	0.521 ± 0.103	0.281 ± 0.024	NS	<0.01	0.548 ± 0.188	0.509 ± 0.084	0.215 ± 0.019	NS	<0.01
Bone marrow	0.152 ± 0.045	0.131 ± 0.030	0.052 ± 0.011	NS	<0.01	0.113 ± 0.027	0.135 ± 0.039	0.042 ± 0.008	NS	<0.01
Liver	0.184 ± 0.018	0.165 ± 0.018	0.192 ± 0.029	NS	NS	0.142 ± 0.019	0.143 ± 0.011	0.156 ± 0.016	NS	NS
Kidney	1.827 ± 0.140	1.696 ± 0.242	2.176 ± 0.480	NS	NS	1.842 ± 0.296	1.878 ± 0.229	2.521 ± 0.203	NS	<0.01

*Group A vs. group B.
†Group B vs. group C.
‡Group D vs. group E.
§Group E vs. group F.
NS = not statistically significant.
Data are shown as (%ID/g) × kg.

kg), compared with that in the corresponding control group (group B, 0.022 ± 0.003 [%ID/g] × kg) (Fig. 1; Table 2). No significant change in the accumulation of ^{99m}Tc-annexin V in tumors was detected in the rats administered cold annexin V after cyclophosphamide treatment (group D, 0.026 ± 0.002 [%ID/g] × kg), compared with that in the corresponding control group (group E, 0.026 ± 0.0024 [%ID/g] × kg) (Fig. 1; Table 2). There were no significant differences in ^{99m}Tc-annexin V accumulation in other tissues, including the thymus, spleen, and bone marrow, between groups A and B and between groups D and E.

DISCUSSION

The results of these experiments demonstrate that accumulation of ^{99m}Tc-annexin V in tumors is not significantly affected by treatment with cold annexin V before or after chemotherapy. Consequently, it appears that the ability of radiolabeled ^{99m}Tc-annexin V to concentrate in tumors remains unchanged in the presence of marked increases in the circulating level of annexin V.

In the clinical evaluation of tumor response to therapy using ^{99m}Tc-annexin V, imaging is usually performed at baseline, to determine the degree of apoptosis in the untreated state, and after 1 or 2 treatments, to determine whether the therapy is efficacious. In the repetitive detection of apoptosis with ^{99m}Tc-annexin V, however, the specific binding of annexin V to phosphatidylserine might affect the subsequent detection of apoptosis with this compound. Our preliminary imaging study with ^{99m}Tc-annexin V suggested prolonged retention of annexin V on phosphatidylserine expressed by tumor cells. Groups A and B were compared to elucidate the feasibility of ^{99m}Tc-annexin V for imaging of tumors in subjects before initiation of chemotherapy and immediately after a single dose of chemotherapy. On the other hand, groups D and E were compared to elucidate the feasibility of ^{99m}Tc-annexin V for imaging of tumors in subjects repetitively after chemotherapy. The present results indicate that repetitive detection of apoptosis with ^{99m}Tc-annexin V is feasible for both imaging protocols. Our study also showed that injection of cold annexin V did not significantly affect accumulation of ^{99m}Tc-annexin V in tumors. In addition, there was no change in tracer concentration in the thymus, spleen, or bone marrow, where apoptosis appeared to be induced by the cyclophosphamide treatment. These results further support the feasibility of repetitive detection of apoptosis using ^{99m}Tc-annexin V.

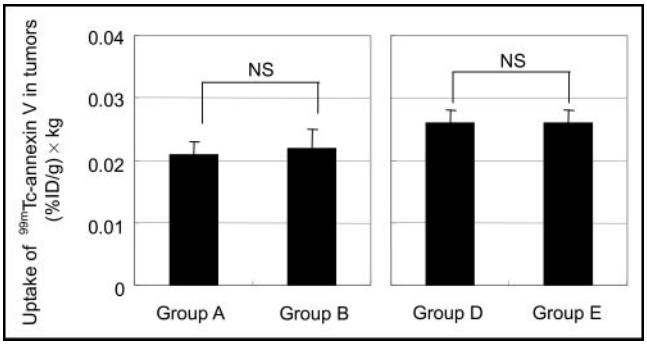

FIGURE 1. Uptake of ^{99m}Tc-annexin V in tumors in rats inoculated with hepatoma cells. NS = not statistically significant.

A dose of 20 μg/kg was selected as the treatment dose of unlabeled (cold) and labeled annexin V, considering the clinical doses used in ^{99m}Tc-annexin V imaging (*10*). Accumulation of ^{99m}Tc-annexin V in tumors and in other tissues was not affected by previous administration of cold annexin V. The amounts of cold annexin V given (20 μg/kg) were probably far below the amount needed to saturate available binding sites on the tumor. It is reported that doses of 300–1,000 μg/kg were needed to produce measurable anticoagulation in vivo in rats (*11*). The fact that anticoagulation requires that a significant fraction of the membrane surface be occupied by annexin V provides some indication of the amount of annexin V that would be needed to saturate available binding sites on tumors. Higher doses of annexin V might affect the accumulation of ^{99m}Tc-annexin V in tumors and other tissues, although it is unlikely that such higher doses of annexin V are used in clinical imaging with ^{99m}Tc-annexin V. It is also important to consider the phosphatidylserine-expression kinetics in relation to the amount of annexin V administered to rats, since phosphatidylserine expression is regarded as a dynamic process. The evidence with annexin V suggested that there are at least 2 peaks of phosphatidylserine expression, one occurring early, within hours of the initiation of chemotherapy, and another probably 24–72 h after the completion of treatment (*12*). Thus, in our study, it is expected that phosphatidylserine is significantly expressed throughout the time studied (24–48 h after treatment). The kinetics of phosphatidylserine expression may not be a responsible factor in the present results.

In the present study, we used annexin V-117 labeled with ^{99m}Tc. Several chelation sites have been proposed for radiolabeling annexin V with ^{99m}Tc (*4,6,9,10*). Annexin V-117, a mutant molecule of annexin V with a high affinity for membrane phosphatidylserine, was produced by expression in *E. coli* and could be used for imaging of cyclophosphamide-induced apoptosis in vivo (*9*). In our untreated rats (groups C and F), the concentrations of ^{99m}Tc-annexin V-117 in the blood and tissues were relatively lower than those of ^{99m}Tc-hydrazinonicotinamide (HYNIC)-annexin V (*7*) and ^{99m}Tc-ethylenedicysteine (EC)-annexin V (*6*), although the biodistribution pattern of ^{99m}Tc-annexin V-117 was similar to those of ^{99m}Tc-HYNIC-annexin V and ^{99m}Tc-EC-annexin V. Clearance of ^{99m}Tc-annexin V-117 may be more rapid than that of ^{99m}Tc-HYNIC-annexin V (*9*). On the other hand, cyclophosphamide (groups B and E) treatment significantly increased the accumulation of ^{99m}Tc-annexin V in the tumor, thymus, spleen, and bone marrow, further confirming the ability of ^{99m}Tc-annexin V-117 to detect cyclophosphamide-induced apoptosis (*9*).

CONCLUSION

Our results demonstrate the feasibility of ^{99m}Tc-annexin V imaging for repetitive detection of apoptosis, which is highly required in clinical evaluation of tumor response to therapy.

ACKNOWLEDGMENTS

The authors thank Dr. Futoshi Okada of the Division of Cancer Biology, Institute for Genetic Medicine, Hokkaido University, for generously providing tumor cells. The authors are grateful to Professors Shinzo Nishi, Kazuo Miyasaka, and Toshiyuki Ohnishi of the Central Institute of Isotope Science, Hokkaido University, for supporting this work. The authors thank Drs. Koutaro Suzuki, Hidenori Katsuura, Hidehiko Omote, and Hiroshi Arai for assistance. This work was supported in part by a grant from the Japanese Foundation for Multidisciplinary Treatment of Cancer and by a grant from the Association for Nuclear Technology in Medicine.

REFERENCES

1. Thompson BC. Apoptosis in the pathogenesis and treatment of disease. *Science.* 1995;267:1456–1462.
2. Thiagarajan P, Tait JF. Binding of annexin V/placental anticoagulant protein I to platelets: evidence for phosphatidylserine exposure in the procoagulant response of activated platelets. *J Biol Chem.* 1990;265:17420–17423.
3. van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry.* 1998;3:1–9.
4. Blankenberg FG, Katsikis PD, Tait JF, et al. In vivo detection and imaging of phosphatidylserine expression during programmed cell death. *Proc Natl Acad Sci USA.* 1998;95:6349–6354.
5. Milas L, Stephens LC, Meyn RE. Relation of apoptosis to cancer therapy. *In Vivo.* 1994;8:665–673.
6. Yang DJ, Azhdarinia A, Wu P, et al. In vivo and in vitro measurement of apoptosis in breast cancer cells using ^{99m}Tc-EC-annexin V. *Cancer Biother Radiopharm.* 2001;16:73–83.
7. Mochizuki T, Kuge Y, Zhao S, et al. Detection of apoptotic tumor response in vivo after a single dose of chemotherapy with ^{99m}Tc-annexin V. *J Nucl Med.* 2003;44:92–97.
8. Belhocine T, Steinmetz N, Hustinx R, et al. Increased uptake of the apoptosis-imaging agent ^{99m}Tc recombinant human annexin V in human tumors after one course of chemotherapy as a predictor of tumor response and patient prognosis. *Clin Cancer Res.* 2002;8:2766–2774.
9. Tait JF, Brown DS, Gibson DF, Blankenberg FG, Strauss HW. Development and characterization of annexin V mutants with endogenous chelation sites for ^{99m}Tc. *Bioconjug Chem.* 2000;11:918–925.
10. Kemerink GJ, Boersma HH, Thimister PW, et al. Biodistribution and dosimetry of ^{99m}Tc-BTAP-annexin-V in humans. *Eur J Nucl Med.* 2001;28:1373–1378.
11. Romisch J, Seiffge D, Reiner G, Paques EP, Heimburger N. In-vivo antithrombotic potency of placenta protein 4 (annexin V). *Thromb Res.* 1991;61:93–104.
12. Blankenberg F. To scan or not to scan, it is a question of timing: technetium-99m-annexin V radionuclide imaging assessment of treatment efficacy after one course of chemotherapy. *Clin Cancer Res.* 2002;8:2757–2758.