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30. We thank L. Luo and Y. N. Jan for the transgenic strains carrying *Drac1* and *Dcdc42* derivatives, T. Uemura for the *elav-GAL4* strain, A. Nose for monoclonal antibody 1D4, A. DiAntonio and E. Giniger for the plasmids containing the *elav* promoter and *kinesin* gene, respectively, C.-S. Yoon for technical instruction, and S. Yoshida, A. Chiba, and all members of our department for comments and discussions. Supported by a Grant-in-Aid for Priority Area from the Ministry of Education, Science, Sports and Culture and the research grant for Nervous and Mental Disorders from the Ministry of Health and Welfare of Japan.

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Tumor Infarction in Mice by Antibody-Directed Targeting of Tissue Factor to Tumor Vasculature

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Selective occlusion of tumor vasculature was tested as a therapy for solid tumors in a mouse model. The formation of blood clots (thrombosis) within the tumor vessels was initiated by targeting the cell surface domain of human tissue factor, by means of a bispecific antibody, to an experimentally induced marker on tumor vascular endothelial cells. This truncated form of tissue factor (tTF) had limited ability to initiate thrombosis when free in the circulation, but became an effective and selective thrombogen when targeted to tumor endothelial cells. Intravenous administration of the antibody-tTF complex to mice with large neuroblastomas resulted in complete tumor regressions in 38 percent of the mice.

As a strategy for cancer therapy, the use of immunoconjugates that selectively occlude the vasculature of solid tumors offers several theoretical advantages over immunoconjugates that target tumor cells directly. First, because tumor cells depend on a blood supply, local interruption of the tumor vasculature will produce an avalanche of tumor cell death (*1*). Second, the tumor vascular endothelium is in direct contact with the blood, whereas the tumor cells themselves are outside the bloodstream and, for the most part, are poorly accessible to immunoconjugates (*2*). Third, tumor vascular endothelial cells are not transformed and are unlikely to acquire mutations that render them resistant to therapy.

We explored the feasibility of treating solid tumors by targeting human tissue factor (TF) to tumor vascular endothelium in a mouse model. TF is the major initiating receptor for the thrombogenic (blood coagulation) cascades (*3*). Assembly of cell surface TF with factor VII/VIIa generates the functional TF:VIIa complex. This complex rapidly activates the serine protease zymogens factors IX and X by limited proteolysis, leading to the formation of thrombin and, ultimately, a blood clot. A recombinant form of TF has been constructed that contains only the cell surface domain (*4*). This truncated TF (tTF) is a soluble protein with a factor X–activating activity that is about five orders of magnitude less than that of native transmembrane TF in an appropriate phospholipid membrane environment (*5*). This is because the TF:VIIa complex binds and activates factors IX and X far more efficiently when associated with a negatively charged phospholipid surface (*5*, *6*). We reasoned that, by using an antibody to target tTF to tumor vascular endothelium, the tTF would be brought into proximity with a cell surface so as to recover in part its native function and locally initiate thrombosis. Such an antibody-tTF conjugate (or "coaguligand") would selectively thrombose tumor vasculature.

To test this concept, we used a mouse model in which the tumor vascular endothelium expresses a marker that is lacking on the normal vascular endothelium (*7*). Naturally occurring markers of tumor vascular endothelium have not been identified in mice, although some strong candidates have been identified for humans (see below). In our model, C1300(Muγ) mouse neuroblastoma cells that have been stably transfected with the murine interferon-γ (IFN-γ) gene are grown as a solid subcutaneous tumor in BALB/c *nu/nu* mice. The IFN-γ secreted by the tumor cells induces local expression of major histocompatibility complex class II antigens (I-A^d and I-E^d) on the tumor vascular endothelium. Class II antigens are absent from normal vascular endothelium in mice, although they are present on B lymphocytes, monocytes, and some epithelial cells.

To target tTF to I-A^d on tumor vascular

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endothelium, we prepared a bispecific antibody with the Fab′ arm of the B21-2 antibody, specific for I-A^d, linked to the Fab′ arm of the 10H10 antibody, specific for a noninhibitory epitope on the C-module of tTF (8). This bispecific antibody, B21-2/10H10, mediated the binding of tTF in an antigen-specific manner to I-A^d on A20 mouse B-lymphoma cells in vitro (Fig. 1A). When mouse plasma was added to A20 cells to which tTF had been bound by B21-2/10H10, it coagulated rapidly. Fibrin strands were visible 36 s after the addition of plasma to antibody-treated cells, as compared with 164 s when plasma was added to untreated cells (Fig. 1B). This enhanced coagulation was observed only when tTF was bound to the cells; no effect on coagulation time was seen with cells incubated with tTF alone, with homodimeric F(ab′)$_2$, with Fab′ fragments, or with tTF plus bispecific antibodies that had only one of the two specificities needed for binding tTF to A20 cells.

There was a linear relation between the logarithm of the number of tTF molecules bound to the cells and the rate of plasma coagulation by the cells (Fig. 1C). In the presence of cells alone, plasma coagulated in 190 s, whereas at 300,000 molecules of tTF per cell, the coagulation time was 40 s. Even with only 20,000 molecules per cell, coagulation was faster (140 s) than with untreated cells. These in vitro experiments showed that the thrombogenic potency of tTF is enhanced by cell surface proximity mediated through antibody-directed binding to class II antigens on the cell surface.

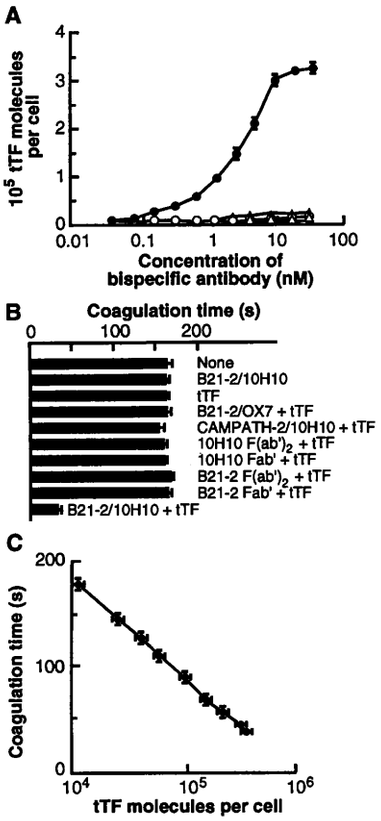

Fig. 1. Effect of the binding of tTF to A20 cells on plasma coagulation. (**A**) Binding of tTF to A20 cells by means of a bispecific antibody. A20 cells (10^5 cells, 100 μl) were incubated with antibodies B21-2/10H10 (●), CAMPATH-2/10H10 (△), B21-2/OX7 (▲), or phosphate-buffered saline (○) plus an excess of ^{125}I-labeled tTF for 1 hour at 4°C in the presence of 0.2% (w/v) sodium azide and bovine serum albumin (2 mg/ml). The cells were centrifuged through a mixture of phthalate oils (*25*). The number of bound tTF molecules per cell was calculated from the radioactivity associated with the pellet. (**B**) Induction of coagulation by cell-bound tTF. A20 cells (10^5 cells, 100 μl) were incubated with antibodies (0.33 μg) and tTF (0.17 μg) for 1 hour at 4°C. Calcium chloride (12.5 mM) and citrated mouse plasma were added to the cells, and the time until the first fibrin strands formed was recorded. (**C**) Relation between the number of bound tTF molecules and plasma coagulation time. A20 cells (10^5 cells, 100 μl) were incubated with varying concentrations of B21-2/10H10 plus an excess of tTF for 1 hour at 4°C in the presence of sodium azide and were then washed and warmed to 37°C. Calcium chloride (12.5 mM) and citrated mouse plasma [a different batch from that in (B)] were added to the cells, and the time until the first fibrin strands formed was recorded. The number of tTF molecules bound to the cells was determined in a parallel experiment with ^{125}I-labeled tTF. Values (±SD) represent the means of three measurements.

A histological study was performed to determine whether intravenous administration of the B21-2/10H10·tTF coaguligand induced selective thrombosis of tumor vasculature in mice bearing subcutaneous C1300(Muγ) neuroblastomas 0.8 to 1.0 cm in diameter (9) (Fig. 2). Within 30 min, all vessels throughout the tumor were thrombosed, containing occlusive platelet aggregates, packed erythrocytes, and fibrin. At this time, tumor cells were histologically indistinguishable from tumor cells of untreated mice. After 4 hours, however, there were signs of tumor cell injury. The majority of tumor cells had separated from one another and had pyknotic nuclei, and the tumor interstitium commonly contained erythrocytes. By 24 hours, the tumor showed advanced necrosis, and by 72 hours, the entire central region of the tumor had condensed into amorphous debris. In contrast, there was no visible thrombosis of tumor vessels in mice 30 min after injection with equivalent quantities of tTF alone or tTF in combination with control bispecific antibodies (OX7/10H10, CAMPATH-2/10H10, or B21-2/OX7) that had only one of the two specificities needed for binding of tTF to I-A^d. Similarly, no thromboses were found in nontransfected C1300 tumors, where the endothelium lacks I-A^d.

These experiments indicated that the predominant occlusive effect of the B21-2/10H10·tTF coaguligand on tumor vessels is

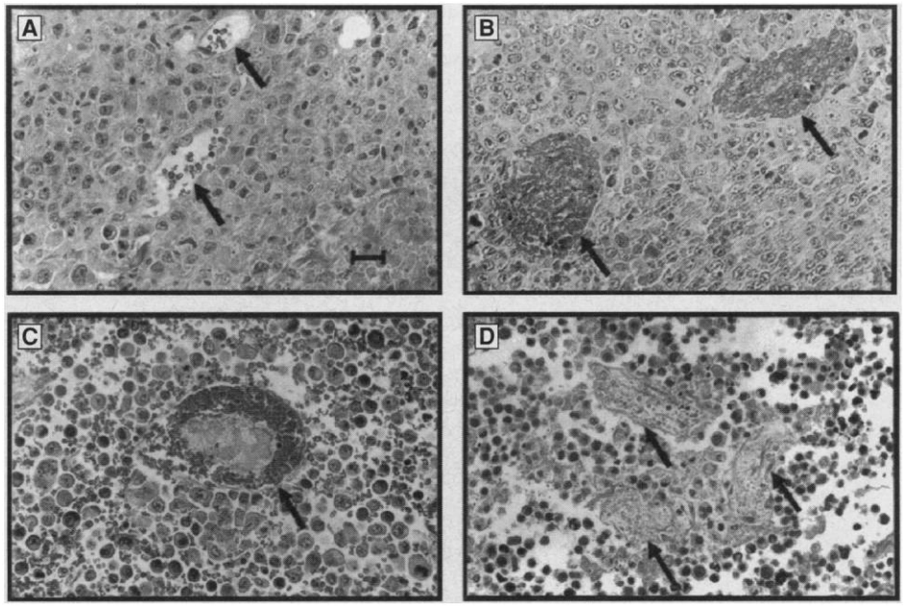

Fig. 2. Histologic analysis of neuroblastomas in mice treated with B21-2/10H10·tTF coaguligand. (**A**) Before injection (0 hours), the blood vessels are intact and the tumor cells appear normal. (**B**) At 0.5 hours, the blood vessels throughout the tumor are thrombosed and the tumor cells are normal. (**C**) At 4 hours, there are dense thrombi in all tumor vessels and the tumor cells are separating from one another, undergoing nuclear pyknosis and cytolysis. Erythrocytes are present in the tumor interstitium. (**D**) At 24 hours, there is advanced necrosis uniformly throughout the tumor. Arrows indicate blood vessels. Scale bar, 50 μm.

mediated through binding to class II antigens on tumor vascular endothelium. Nevertheless, a nonspecific thrombotic action of tTF was discernible in tumor vessels at later times; in tumors from mice that had been injected 24 hours previously with tTF alone or tTF mixed with the control bispecific antibody OX7/10H10, an average of 40% and 60% of the vessels were thrombosed, respectively. These were most prevalent in the tumor core. It is possible that the resident thrombogenic activity of tumor vasculature (*10*) renders these vessels more susceptible to thrombosis even by untargeted tTF. Alternatively, enhanced procoagulant changes might have been induced by the tumor-derived IFN-γ. Coaguligand treatment was well tolerated (*11*); mice lost no weight and retained a normal appearance and level of activity. Neither thrombi nor histological abnormalities were found in the liver, kidney, lung, intestine, heart, brain, adrenals, pancreas, or spleen from the tumor-bearing mice 30 min or 24 hours after administration of coaguligand or free tTF.

We next investigated whether intravenous administration of the B21-2/10H10·tTF coaguligand could inhibit the growth of large tumors (diameter 0.8 to 1.0 cm) in mice (9). The pooled results from three separate experiments (Table 1 and Fig. 3) indicate that 38% (8 of 21) of mice receiving B21-2/10H10·tTF coaguligand had complete tumor regressions lasting 4 months or more. A further 24% (5 of 21) had reductions in tumor volume in excess of 50%. These antitumor effects were significantly greater than for all other treatment groups ($P < 0.05$).

The antitumor effect of the B21-2/10H10·tTF coaguligand was attributable in part to a nontargeted effect of tTF. Tumors in mice receiving tTF alone or mixed with control bispecific antibodies (CAMPATH-2/10H10 or B21-2/OX7) grew significantly more slowly than did tumors in mice receiving antibodies or saline alone ($P < 0.05$). The nontargeted effect of tTF on tumor growth presumably derives from its slight residual thrombogenic activity coupled with the endogenous thrombogenic activity of tumor vessels. However, the nontargeted effect of tTF was weak compared with the coaguligand effect. No mice receiving tTF alone had complete tumor regressions, and only 8% (1 of 12) had a partial remission.

In mice that did not show complete tumor regression after B21-2/10H10·tTF coaguligand treatment, the tumors grew back from a surviving microscopic rim of cells at the periphery of the tumor. Immunohistochemical examination of these tumors revealed that the vascular endothelium at the invading edge of the tumors lacked detectable class II antigens, consistent with a lack of thrombosis of these vessels by the coaguligand, which permitted local tumor cell survival (*12*). Conceivably, coadministration of a drug acting on the tumor cells themselves might improve efficacy, as we observed with another antivascular therapy (*13*, *14*). We previously demonstrated that a powerfully cytotoxic ricin A-chain immunotoxin directed against the tumor cells themselves was virtually devoid of antitumor activity when administered to mice with large C1300(Muγ) tumors (*13*, *14*). The lack of activity was a result of the inability of the immunotoxin to gain access to tumor cells in large tumor masses, thus attesting to the comparative effectiveness of coaguligand therapy.

Our experiments illustrate the therapeutic potential of selective initiation of the blood coagulation cascade in tumor vasculature. For clinical application, this strategy will require the identification of target molecules (antigens, receptors) that are present

Table 1. Antitumor effects of B21-2/10H10·tTF coaguligand. The tumor growth index is the ratio of mean tumor volume on day 14 to mean tumor volume on day 0. CR, complete regression; PR, partial remission (>50% decrease in initial tumor volume); NR, no response (<50% decrease in initial tumor volume). Two-tailed *P* values are for differences in tumor volume (day 14) by the Mann-Whitney rank sum test; NS, not significant.

Treatment	*n*	Mean tumor volume (mm^3)		Tumor growth index	Response (%)			*P*	
		Day 0	Day 14		CR	PR	NR	Versus saline	Versus tTF
Saline	27	282	1643	5.8	4	0	96	–	0.05
B21-2/10H10·tTF	21	270	466	1.7	38	24	38	<0.0001	0.0005
tTF	12	285	1054	3.7	0	8	92	0.05	–
B21-2/10H10	13	289	1346	4.7	0	8	92	NS	NS
B21-2/OX7 + tTF	14	293	1027	3.5	7	0	93	0.01	NS
CAMPATH-2/10H10·tTF	8	285	975	3.4	0	0	100	0.002	NS

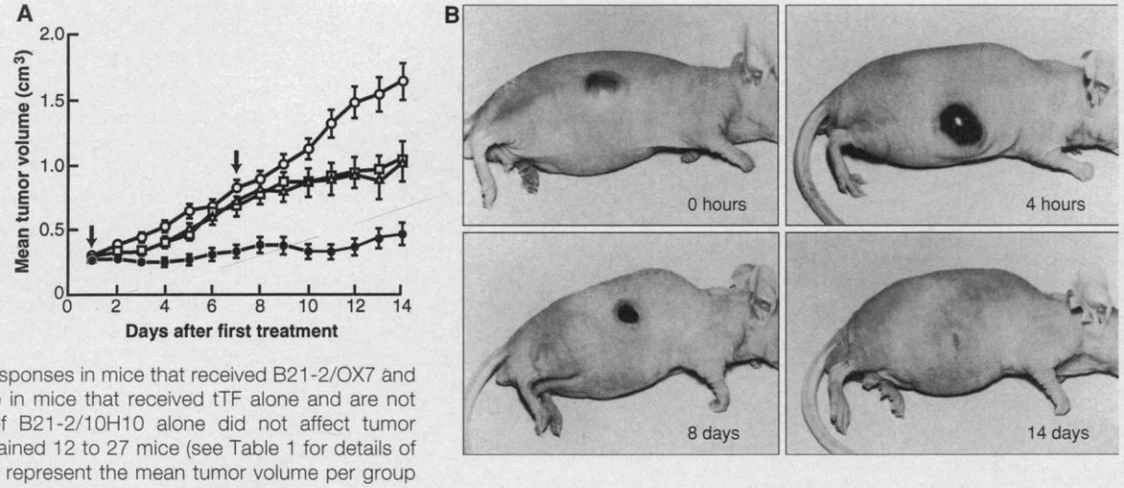

Fig. 3. Tumor regression induced by B21-2/10H10·tTF coaguligand. (**A**) Mice with C1300-(Muγ) tumors (diameter 0.8 to 1.0 cm) were given two intravenous injections of B21-2/10H10·tTF coaguligand spaced 6 days apart (arrows) (●). Mice in control groups received equivalent doses of tTF alone (□), CAMPATH-2/10H10 plus tTF (△), or phosphate-buffered saline (○). Tumor responses in mice that received B21-2/OX7 and tTF were similar to those in mice that received tTF alone and are not shown. Administration of B21-2/10H10 alone did not affect tumor growth. Each group contained 12 to 27 mice (see Table 1 for details of individual groups). Points represent the mean tumor volume per group (±SEM). (**B**) Gross appearance of subcutaneous tumors after treatment with B21-2/10H10·tTF coaguligand. At the time of treatment (0 hours) the tumor was pink, indicating florid vascularization. After 4 hours, the tumor appeared bruised and blackened. Over the next 8 days the tumor collapsed. By day 14, only fibrous scar tissue was visible in many of the mice.

at sufficient density on the surface of tumor vascular endothelium but absent from normal vascular endothelium (*15*). Promising candidate molecules for humans include endoglin (*16*), endosialin (*17*), an endoglin-like molecule (*18*), a fibronectin isoform (*19*), an osteosarcoma-related antigen (*20*), CD34 (*21*), collagen type VIII (*22*), the vascular endothelial cell growth factor (VEGF) receptors (*23*), and VEGF itself (*24*). The induction of tumor infarction by targeting a thrombogen to these or other tumor endothelial cell markers represents an intriguing approach to the eradication of primary solid tumors and vascularized metastases.

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Geographic Distribution of Endangered Species in the United States

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Geographic distribution data for endangered species in the United States were used to locate "hot spots" of threatened biodiversity. The hot spots for different species groups rarely overlap, except where anthropogenic activities reduce natural habitat in centers of endemism. Conserving endangered plant species maximizes the incidental protection of all other species groups. The presence of endangered birds and herptiles, however, provides a more sensitive indication of overall endangered biodiversity within any region. The amount of land that needs to be managed to protect currently endangered and threatened species in the United States is a relatively small proportion of the land mass.

Previous studies have shown that, on a continental scale, the distributions of well-studied taxa can act as surrogates or indicators for the distribution of poorly studied taxa (*1*–*4*). In contrast, studies of the distribution of "hot spots" of diversity for various taxa within the British Isles suggest that there is very little correlation between the distributions of different taxonomic groups (*5*, *6*). To date, however, no such analysis has been done on a continental or national scale for those species most likely to vanish in the foreseeable future, that is, endangered species. If significant correlations occur in the geographic distributions of different groups of endangered species, it may be possible to use a few well-studied groups as indicators for the purposes of delineating protected areas for other poorly known taxa. The extent to which endangered species are concentrated in hot spots of potential extinctions and the extent to which hot spots for different groups overlap will influence the strategies we adopt to avert species extinctions and the impact of those strategies on other human activities (*7*, *8*). If endangered species are highly concentrated, then fewer areas are likely to experience conflicts between species protection and other activities.

In this study, we used a database of threatened and endangered species in the United States to examine patterns in the geographic distribution of imperiled species (*9*). The database lists the counties of occurrence of all plants and animals protected under the federal Endangered Species Act in the 50 states, plus all species, subspecies, and populations proposed for protection under that statute as of August 1995 (a total of 924 species in 2858 counties). We grouped the species by state, county, and species group (amphibians, arachnids, birds, clams, crustacea, fish, insects, mammals, plants, reptiles, and snails) and then generated dis-

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