activity in the intercalated cells (*9*), another sign that these cells play a potential role in the reabsorption of bicarbonate and in the secretion of hydrogen ions (under normal conditions). It is thus reasonable to propose that anion transport in intercalated cells is catalyzed by an isoform of band 3, which is concentrated along the basolateral plasma membrane of these cells. The observation that kidney band 3 reacts with antibodies to both the membrane-spanning and cytoplasmic domain of rat and human band 3 indicates a high degree of relationship between erythroid and kidney band 3.

Ankyrin and spectrin, the membrane-skeletal proteins associated with band 3 in erythrocytes, are also present in kidney epithelial cells. Antibodies to ankyrin reacted strongly with the basolateral plasma membrane of different epithelial cell types in the nephron (most intensely at basal plasmalemmal infoldings of the pars recta of distal tubules). Importantly, in the collecting ducts strong immunostaining specific for both the band 3–binding (M_r ~90,000) and spectrin-binding (M_r ~72,000) domain of human erythrocyte ankyrin was precisely colocalized with the membrane sites of band 3 in the intercalated cells (Fig. 2a). The strongest immunostain was found at the basal rather than at the lateral cell membrane. The luminal membrane of intercalated cells and the cell membrane of the principal collecting duct cells were only faintly labeled. Western blot analysis of membranes from perfused kidneys revealed isoforms of ankyrin of M_r 190,000 and 210,000. Ankyrin analogs of identical molecular weight have been described from brain (*10*). Antibodies to brain spectrin stained all tubular and ductal cells along the whole membrane (Fig. 2c), a pattern that has also been demonstrated for spectrin analogs in other types of epithelial cells (*11*).

The codistribution of kidney band 3 with ankyrin and spectrin suggests a lateral association between band 3 and the membrane cytoskeleton. The association between integral membrane proteins and the cytoskeleton may serve as a general molecular way of restricting the lateral mobility of membrane proteins and for localizing them in a nonrandom fashion at specialized regions of the cell surface. The mechanisms responsible for concentrating ankyrin and band 3 at the basolateral membrane remain to be determined. Band 3, once inserted, would be restricted in its lateral diffusion by linkage to ankyrin and spectrin. It will be important in future work to purify the kidney anion transport protein and to determine if it has anion transport characteristics similar to those of erythrocyte band 3 and if it has an ankyrin binding site.

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Tissue Plasminogen Activator Reduces Neurological Damage After Cerebral Embolism

Abstract. *Intravenous administration of tissue plasminogen activator immediately after the injection of numerous small blood clots into the carotid circulation in rabbit embolic stroke model animals caused a significant reduction in neurological damage. In vitro studies indicate that tissue plasminogen activator produced substantial lysis of clots at concentrations comparable to those expected in vivo, suggesting that this may be the mechanism of action of this drug. Drug-induced hemorrhages were not demonstrable. Tissue plasminogen activator may be of value for the immediate treatment of embolic stroke.*

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Stroke is currently the third most frequent cause of death in the United States. The three main causes of stroke are thrombosis, embolism, and hemorrhage. At present, there is no generally accepted specific therapy for thrombosis. Anticoagulation with heparin is accepted as effective in prophylaxis of cerebral embolism of cardiac origin and possibly for stroke in evolution (progressive increases in neurological deficits during the first 24 to 72 hours after the onset of ischemic strokes) (*1*). Even in these most treatable strokes, heparinization merely prevents subsequent infarction and does nothing to reverse damage produced by the initial event. Both thrombosis and embolism are potentially treatable by thrombolytic therapy. However, thrombolytic therapy with streptokinase or urokinase has been unsuccessful because intravenous administration has caused severe hemorrhagic side effects (*2*). Moreover, local injection of these drugs into the appropriate occluded artery requires catheterization, and such a procedure is so time-consuming that irreversible tissue damage may occur long before effective thrombolysis can be achieved. Consequently, it is clear that there is need for more effective methods of stroke therapy.

Tissue plasminogen activator (tPA) appears to have substantial potential for treatment of thromboembolic strokes. Studies of its efficacy in the treatment of coronary artery thrombosis (*3*) indicate that tPA may be of substantial benefit in treatment of the most common types of strokes, particularly since tPA can be given intravenously and appears to cause minimal risk of peripheral hemorrhagic complications. (*4*).

One of the main impediments to estab-

lishing the efficacy of any form of stroke therapy is the highly diverse nature of strokes. The brain has a very complex structure, and its vascular supply is quite variable. Consequently, in order to prove that a given form of stroke therapy is effective in humans, it is usually necessary to conduct a very large study. This degree of diversity is true of most animal models as well. Although several embolic stroke models have been devised (*5*), such models have not been widely used because, in addition to the problems of defining the location and severity of damage produced during a stroke, there is also the problem of predetermining precisely where embolic material will lodge. In general, such unpredictability of the cerebral infarct is intolerable in screening studies, particularly when the dependent variables are infarct size and location. We have developed a technique that does not require reproduction of these end points and allows us to demonstrate quickly and at relatively low cost the efficacy of tPA for reduction of damage produced by embolic stroke.

New Zealand White rabbits weighing 2 to 2.5 kg each were anesthetized with ether, the bifurcation of one carotid artery was exposed, and the external carotid was ligated just distal to the bifurcation. A plastic catheter was inserted into the common carotid and secured with ligatures. The incision was closed around the catheter so that its distal end was accessible outside of the animal's neck. The line was filled with heparinized saline and capped with an injection cap. The animals were then allowed to recover from anesthesia for at least 3 hours so that they were awake and appeared to behave normally.

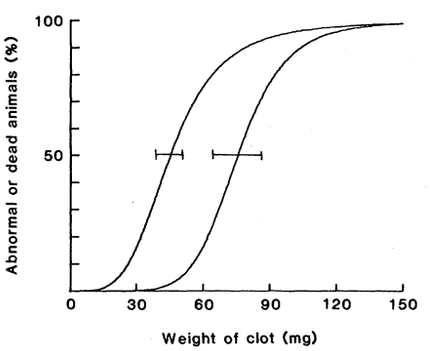

Fig. 1. Percentage of animals dead or severely damaged neurologically as a function of the weight of clots injected into the carotid artery system. The curve on the left shows the fraction of control animals damaged by various doses of clot material. It demonstrates that 50 percent of the animals injected with 45.3 mg of clots will be abnormal or dead at 24 hours after the injection of clots. The curve on the right indicates that, if tPA is infused intravenously after the injection of clots by the schedule indicated in the text, the weight of clots can be increased to 75.7 mg before 50 percent of the treated animals suffer neurological damage or death. The horizontal bar on each curve indicates the standard error at the ED_{50}. The data plotted here are presented in Table 1.

Each day blood was drawn from a donor rabbit and allowed to clot for 2 hours at 37°C. The clot was suspended in calcium-free Dulbecco's phosphate-buffered saline (PBS) solution (*6*) containing 0.1 g of bovine serum albumin per deciliter of solution and fragmented by use of a Polytron (Brinkmann Instruments, Westbury, New York). The fragments were sized by sequential filtration through first a 240-μm^2 screen and then a 104-μm^2 nylon net. The particles were washed with the PBS solution and allowed to settle, and the supernatant was removed by gentle suction. An aliquot of the concentrated particles was then spun at 12,000*g* for 10 minutes. The pellet was weighed to determine the wet weight of the clot particles. Appropriate volumes of the PBS solution were then added to the concentrated particle suspension to allow injection of the required weight of clot particles into the animal. The particle suspension was diluted so that a predetermined weight of particles would be suspended in 3 ml, which was then drawn into a syringe. On each day, one clot suspension was made and all animals received injections from the same suspension. Either two or three pairs of test animals were used each day. Animals were randomly assigned to be either the control or the treatment member of the pair. Control animals received normal saline, and treatment animals were given tPA (Genentech, Inc., South San Francisco, California). By use of this design, any small differences in the sizes or weights of the particles that were prepared each day were obviated by the fact that the paired control and treatment animals received matched clots.

At the time of injection, each animal was restrained and the injection cap was removed to allow the rabbit's own blood to fill the catheter and wash out the heparin solution that was used to keep the catheter patent. The line was then filled with heparin-free normal saline and the injection cap was replaced with care so that no bubbles were present in the catheter or the syringe. An intravenous line was placed in an ear vein and attached to a syringe containing enough tPA solution (0.5 mg/ml) so that the animal would ultimately receive 1 mg/kg. The clot particles were then rapidly injected, and the clot injection system was flushed with 5 ml of normal saline. Within 2 minutes thereafter 20 percent of the tPA solution was injected, and the remainder was infused over a period of 30 minutes with an infusion pump.

After the infusions were complete, the animals were released and replaced in their home cages. They were then observed for level of neurological function or any evidence of seizures. Neurological function was scored on a three-point scale: (i) normal activity; (ii) abnormal activity (this grade included any unequivocal behavioral disturbances such as a greatly reduced level of spontaneous activity, inability to stand, markedly uncoordinated movements, and gross nystagmus); or (iii) death.

The animals were observed for 2 hours after the end of treatment. They were randomly coded, and neurological function grades were independently assessed by two observers 24 hours after treatment. One of the observers had not been present at the time when the animals were treated and was thus blinded as to the treatment regimen. There were no intraobserver discrepancies in the grades that were assigned. Animals that had gross behavioral disturbances were killed after 24 hours and their brains were removed for neuropathological examination. Normal animals were maintained for 7 days, and then brains from

Table 1. Effects of tPA treatment on the neurological status of rabbits. Animals were given intracarotid injections of varying weights of clot particles and were treated with either normal saline (control) or intravenous tPA starting within 2 minutes after the injection of clots. The number of animals exhibiting each neurological grade is shown. Classification of neurological status is defined in the text.

Weight of clots (mg)	Neurological status	
	Number normal	Number abnormal or dead
	Control	
15	2	0
30	3	1
36	0	1
42	2	0
45	2	0
48	1	2
54	1	3
60	1	2
72	0	2
90	0	2
150	0	1
	tPA (1 mg/kg)	
42	2	0
48	3	0
54	1	1
60	2	0
72	2	0
90	0	2
150	0	2

all tPA-treated animals and controls representative of each neurological function status were collected for histological study.

Animals were killed by asphyxiation with CO_2. The brains were removed and immersion-fixed in 10 percent phosphate-buffered formalin for at least 2 weeks. They were cut in the coronal plane (midfrontal, midparietal, and midcerebellar levels) and embedded in paraffin, and 6-μm sections were stained with hematoxylin and eosin.

Twenty-four rabbits were given from 42 to 72 mg of clots, representing approximately 2100 to 3600 clots per animal. Of 12 control animals, 3 were grossly abnormal neurologically and 4 were dead 24 hours after injection of the clots. Of the 12 other animals given matching amounts of clots, tPA was successfully administered to 11 (in the remaining animal the intravenous line was dislodged and the animal received only a small partial dose of tPA and thus was discarded from further consideration). Of these 11 animals, 10 were classified as normal both at 24 hours and 1 week after the insult (the remaining animal was dead at 24 hours). No seizure activity was observed at any time in either the control or the treated animals. According to Fisher's exact test, there was a significant difference ($P = 0.027$) between tPA-treated and control animals.

To examine the effects of tPA therapy in greater detail, we pooled an unpaired set of animals used to establish the clot dose range with the paired group. Pooling was legitimate because, aside from the pairing procedure, there were no differences in technique. The results are summarized in Fig. 1 and Table 1. Analysis by a quantal dose-response method (*7*) revealed that the average weight of clots required to produce neurological deficits or death (ED_{50}) was 45.3 ± 5.6 mg in the control group; the ED_{50} for tPA-treated animals was 75.7 ± 9.5 mg. According to the t-test, this difference is also significant ($P < 0.01$).

Histological examination of the animals' brains revealed that the 1-week-old embolic infarcts were present at random locations throughout the supratentorial gray matter and cerebellar cortex in most of the tPA-treated animals and controls. There was no qualitative difference in the size, distribution, or cellular characteristics of infarcts between treated and control groups. In animals that died within 24 hours, some microscopic hemorrhages were observed in the brain parenchyma. No large hemorrhages were observed in any of the animals. All animals had small amounts of blood in the subarachnoid and intraventricular spaces, and occasional subarachnoid vessels were completely occluded with clots. This blood was present in animals in both the control and the treatment groups and was present in animals that died within 24 hours as well as those that were killed 1 week after embolization. Thus, the subarachnoid and intraventricular blood appears to be a feature of the model that does not influence observed neurological function and is unrelated to tPA therapy.

In an earlier preliminary trial, 0.3 mg of tPA per kilogram was injected into treatment animals at a constant rate over 15 minutes starting within 2 minutes after injection of 45 mg of clots. In that trial, seven of nine controls were normal whereas five of eight treated animals were normal. This difference was not significant.

To establish that tPA was able to lyse the artifically made clots that were injected, we conducted an in vitro study of the effect of tPA concentration on the rate of clot lysis. The results (Fig. 2) indicate that clot lysis occurred at low tPA concentrations comparable with the blood concentrations one would expect from the injected dose (*8*) and that lysis was substantial by 15 minutes.

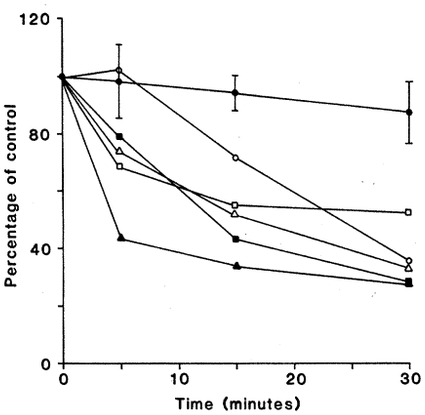

Fig. 2. Dissolution of clot particles by tPA as a function of time. Various amounts of tPA were added to test tubes containing 40 mg (wet weight) of clot particles suspended in 1 ml of rabbit serum. The mixture was incubated at 37°C, and the reaction was terminated by the addition of an excess of aprotinin. The contents of each tube were then poured on a preweighted 74-μm^2 wire screen and then washed with 1 ml of PBS buffer on a vacuum manifold so that the remaining large clot particles were trapped on the filter screens. The screens were then dried at 60°C until a stable dry weight was obtained. Filled circles are controls; error bars indicate standard errors ($n = 4$). Other symbols indicate the average of two independent determinations for the following tPA concentrations: ○, 0.5 μg/ml; □, 1 μg/ml; ■, 2 μg/ml; ▲, 6 μg/ml; △, 18 μg/ml.

These studies demonstrate that intravenous administration of tPA was effective in preserving neurological function of rabbits after the injection of numerous small clots into the cerebral arterial circulation. Successful treatment was accomplished with doses that did not appear to cause important cerebral hemorrhagic complications. The histological patterns of damage were similar in both control and tPA-treated animals. Our neuropathological observations were intended to screen for new patterns of damage, and this study was not designed to allow quantitative histological measurements. Until such a detailed investigation is conducted, further conclusions concerning morphology are speculative. Since the preliminary trial of tPA at a lower dose was unsuccessful, the drug effect was, at least to some extent, dose-dependent. It is probable that, in view of the rapid turnover rate of tPA (*9*), this constant infusion rate was inadequate because the drug was degraded before sufficient concentrations of it in the blood could be obtained. The in vitro studies suggest that the beneficial effects were caused by clot lysis that was sufficiently rapid to prevent development of cerebral ischemia.

In this study we also demonstrated the utility of this multiple embolism model for screening the effects of pharmacological agents in the reduction of cerebral ischemic damage. The use of a simplified scale for assessment of neurological function was objective and reproducible, as demonstrated by the concurrence of grading by multiple observers, and there was excellent precision, which was indicated by the quite small error limits on each ED_{50}. Our study also demonstrates that the formal methods of data analysis introduced for use in studies of spinal cord injury (*10*) can be effectively extended to assays of cerebral function, particularly for objective testing of treatment effects.

Thrombolytic therapy with tPA is potentially more attractive than treatment with streptokinase or urokinase because tPA is relatively clot-specific in its activation of the fibrinolytic system; tPA has a high affinity for fibrin and induces the degradation of plasminogen to its active fibrinolytic enzyme, plasmin, essentially only in the presence of fibrin (*11*). Consumption of fibrinogen and a tendency for bleeding is substantially reduced (*4, 12*). This is particularly important in cases of ischemic stroke because hemorrhagic transformation of a bland cerebral infarction may occur and cause increased mortality and morbidity (*13, 14*).

In this study we selected the size of

emboli and the timing of drug administration to increase the likelihood of demonstrating therapeutic efficacy. The apparent safety of the drug in these experimental conditions does not prove that tPA will be safe in cases where larger emboli lodge in the cerebral circulation and hemorrhagic complications may arise after the onset of ischemia. Until such preliminary studies are completed, trials with human patients will be premature. However, the potency ratio for tPA of 1.7 (*15*) suggests that this drug may be beneficial for such urgent medical problems as frequent transient ischemic attacks with threatened infarction and stroke in evolution. Whether further benefits may be derived from treatment with tPA after more severe ischemic damage has already been done remains to be determined.

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14. We are currently engaged in a number of further investigations to examine such factors as the length of time after embolization over which tPA therapy can reduce neurological damage, the effects of varying the size of emboli, and the determination of whether hemorrhagic complications will develop after cerebral ischemic damage is produced. All of these questions can be experimentally approached with the embolic stroke model we have described here.
15. The potency ratio is the ED_{50} of the treated animals divided by the ED_{50} of the controls.

16. We thank Genentech, Inc., for supplying the tPA.

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Cretaceous-Tertiary Extinctions: Alternative Models

Officer and Drake (*1*) appear to present a valid alternative to the large impact model for the Cretaceous-Tertiary extinctions. Their basic premise is that the distribution of iridium (Ir) in some sections can only be explained by an event of relatively long duration. This proposal is supported by a model which suggests that Ir distribution extending beyond an interval expected by bioturbation requires a noninstantaneous source for the siderophiles. They further suggest that evidence favors a mantle (volcanic) origin for these elements. We find that (i) the bioturbation model is inaccurately applied and inadequately explains possible sedimentary effects for any given section, (ii) there is no evidence of prolonged Ir sedimentation at any site, and (iii) the volcanic model, although not positively excluded by the data, is not easily reconciled with the data and remains at best a very low probability alternative to the impact hypothesis.

The bioturbation interval of 11 cm (5 to 6 cm after compaction) used by Officer and Drake applies only to the homogenization interval for surface sediments. As deposition (and burial) proceeds, older sediments continue to be mixed upward. Sediments that are deposited instantaneously (like microtektites or ash) can be spread over tens of centimeters (*2*). Table 1 shows the observed distribution of microtektites (*3*), which are spread over a minimum of 35 cm and an average of 59 cm. Bioturbation is only one process that will affect Ir distribution after a presumed impact. Other potentially important processes include diagenetic mobilization, secondary deposition after transport by bottom currents, and delayed deposition of siderophiles in solution, which have relatively long residence times.

Table 1. Observed distribution of microtektites in eight piston cores (*3*). The duration of the microtektite zones indicates the time interval represented by the length of sediment across which the microtektites have been smeared by bioturbation.

Core	Sedimentation rate ($m/10^6$ years)	Microtektite zone	
		Length (cm)	Duration (years)
RC8-52	5.6	80	145,000
RC8-53	2.6?	40?	155,000?
RC9-137	6.4	40	63,000
RC9-143	5.1	50	98,000
V16-70	2.2	70	320,000
V16-76	15.1	~50	~33,000
V19-153	7.5	35	47,000
V19-297	7.0	90	130,000
Average	7.0	59	130,000

Because there is no independent evidence for prolonged deposition of Ir-rich sediment at the four sites cited by Officer and Drake, the bioturbation argument may not be relevant.

1) Site 465A was grossly disturbed by drilling, and Cretaceous and Tertiary sediments are mixed over an interval of at least 100 cm (*4*). The published profile for this locality does not in any way reflect the original stratigraphy, which cannot be determined in this section.

2) Anomalously high concentrations of Ir have been reported in only 10 cm of the core at site 524 (*5*); the 43 cm cited by Officer and Drake is the distance to the first background analysis.

3) High Ir concentrations below the fish clay in Stevns Klint, Denmark, rely on correction for more than 99 percent $CaCO_3$, but the significance of the procedure is not clear. The highest concentrations measured outside the fish clay are approximately 0.1 ng/g Ir and probably amount to only a few percent of the total Ir in the section at best. It is reasonable to expect some diffusion of siderophiles out of the clay during diagenesis, and there is no evidence for prolonged deposition of large amounts of siderophiles. Moreover, Stevns Klint is a prime example of a lithologic discontinuity, which Officer and Drake state "preclude[s] precise geologic time discrimination." In fact, every known K/T boundary section has a lithologic discontinuity, and that, if we follow the criteria of Officer and Drake, invalidates their time interval estimates.

4) The Brazos River, Texas, section shows irregular peaks of significant amounts of Ir over about 45 cm on top of a thick turbidite-like sediment at the K/T boundary (*6*). This is a shallow shelf environment where lateral transport, reworking, and winnowing of sediments by storm waves is common. The Ir distribution is easily explained in terms of these mechanisms and by bioturbation.

With regard to characteristics of the event that suggest a mantle rather than meteoritic source, we make the following points.

1) The discovery of Ir-bearing particulates from Kilauea (*7*) is important. However, such particulates would be deposited near the source; we cannot imagine a volcanic event capable of worldwide distribution of the spheroidal material common to KT sediments (*8*).