Mechanism of the cardioprotective effect of transforming growth factor $\beta_1$ in feline myocardial ischemia and reperfusion

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ABSTRACT We followed the effects of transforming growth factor $\beta_1$ (TGF-$\beta_1$) in a feline model of myocardial ischemia (1.5 hr) and reperfusion (4.5 hr). Myocardial ischemia followed by reperfusion resulted in severe myocardial injury, endothelial dysfunction, high cardiac myeloperoxidase activity indicative of neutrophil accumulation in the ischemic myocardium, and significant neutrophil adherence to the ischemic coronary endothelium. In contrast, intravenous administration of TGF-$\beta_1$ (20 $\mu$g/kg) 30 min prior to reperfusion significantly attenuated myocardial necrosis (13.8$\% \pm 3.5$% vs. $32.2 \% \pm 2.9$% of area-at-risk, $P < 0.01$) and attenuated endothelial dysfunction ($P < 0.01$ associated with ischemia-reperfusion. Moreover, TGF-$\beta_1$ decreased neutrophil adherence to the ischemic myocardium. These results demonstrate that TGF-$\beta_1$ exerts a significant cardioprotective effect in a feline model of myocardial ischemia and reperfusion. The mechanism of this protective effect appears to relate to endothelial preservation by TGF-$\beta_1$ inhibiting circulating neutrophils from adhering to the endothelium, a critical step in neutrophil-induced reperfusion injury.

Transforming growth factor $\beta$ (TGF-$\beta$) is a homodimeric protein with a molecular size of 25 kDa that exists in several isoforms (1-3). The most commonly appearing form is TGF-$\beta_1$, which acts as a regulatory substance modulating various biological actions (4). TGF-$\beta$ is a ubiquitous molecule, found in virtually all cell types of the cardiovascular system, including cardiac myocytes, vascular smooth muscle, endothelial cells, fibroblasts, and blood cells (i.e., platelets and leukocytes). TGF-$\beta$ binds to a multitude of cell types via specific membrane receptors (5).

TGF-$\beta_1$ has been shown to protect the ischemic-reperfused myocardium in the rat (6). Moreover, TGF-$\beta_1$ was recently shown to also protect against circulatory shock produced by ischemic-reperfused splanchnic visceral organs in the cat (7). In both cases, TGF-$\beta_1$ was shown to preserve endothelial cell function. However, the mechanisms of this endothelial protective effect are not well established. One key aspect of the endothelial dysfunction relates to the preservation of endothelium-derived relaxing factor (EDRF) (6). However, EDRF release can be markedly reduced by adherence of neutrophils to the endothelium and the subsequent release of neutrophil mediators of cell injury, particularly superoxide radicals (8).

The major purposes of this study were to (i) verify if a cardioprotective effect of TGF-$\beta_1$ occurs on reperfusion injury following myocardial ischemia (MI) using a necrosis-based injury method indexed to the area-of-risk and (ii) determine the cellular mechanism of any potential cardio-protective effect relative to endothelial and neutrophil function and their interaction. In this manner, the nature of the cytoprotective effects of TGF-$\beta$ may be better understood.

MATERIALS AND METHODS

Adult male cats (2.9-4.1 kg) were anesthetized with sodium pentobarbital (30 mg/kg, i.v.). An intratracheal cannula was inserted through a midline incision, and all cats were placed on intermittent positive-pressure ventilation (Harvard small animal respirator, Dover, MA). A polyethylene catheter was inserted into the right external jugular vein for supplementary pentobarbital infusion to maintain a surgical plane of anesthesia and for administration of drugs or their vehicle. An additional polyethylene catheter was positioned in the abdominal aorta for measurement of mean arterial blood pressure (MABP) via a Statham P23AC pressure transducer. After a midsternal thoracotomy, the pericardium was incised and a 3.0 silk ligature was placed around the left anterior descending (LAD) coronary artery 8-10 mm from its origin. Standard lead II of the electrocardiogram (ECG) was used to determine heart rate (HR) and ST segment elevation. The pressure-rate index (PRI), an approximation of myocardial oxygen demand, was calculated as the product of MABP and HR divided by 1000.

Experimental Protocol. After completing all surgical procedures, the cats were allowed to stabilize for 30 min, at which time a baseline reading of ECG and MABP was recorded and a 3-ml blood sample was drawn. The blood was centrifuged at 2500 x g for 15 min at 4°C to obtain plasma that was used as a vehicle for TGF-$\beta_1$ administration. MI was produced by tightening the previously placed reversible ligature around the LAD coronary segment to completely occlude the vessel. This was designated as time 0. One hour after coronary occlusion, TGF-$\beta_1$ (20 $\mu$g/kg) or its vehicle (1 ml of plasma) was given i.v. as a bolus. After 1.5 hr of ischemia, the LAD ligature was untied and the ischemic myocardium was reperfused (R) for 4.5 hr. The animals were randomly divided into three major groups of six or seven cats each. These groups included sham MI + R cats receiving TGF-$\beta_1$ and MI + R cats receiving either TGF-$\beta_1$ or its vehicle. Sham MI + R cats were subjected to the same surgical procedures as MI + R cats except that the LAD coronary artery was not occluded.

Myocardial Tissue Analysis. At the end of the experiment, the ligature around the LAD coronary segment was retightened. Twenty milliliters of 0.5% Evans blue was injected over a period of 1 min into the left ventricle to stain the area of...
myocardium that was perfused by the patency coronary arteries. The area-at-risk was thus determined by negative staining. The unstained portion of the myocardium (i.e., total area-at-risk) was separated from the Evans blue stained portion of the myocardium (i.e., area-not-at-risk). The area-at-risk was again sectioned into 1-mm-thick slices and incubated in 0.1% nitroblue tetrazolium in phosphate buffer at pH 7.4 and 37°C for 15 min, as described (9). The nonischemic, ischemic nonnecrotic, and ischemic necrotic tissues were subsequently weighed and the results were expressed as the area-at-risk indexed to the total left ventricular mass, the area of necrotic tissue indexed to the area-at-risk, and the area of necrotic tissue indexed to the total left ventricular mass.

**Isolated Coronary Ring Studies.** LAD and left circumflex (LCX) coronary segments were removed and placed into warmed Krebs–Henseleit (K–H) buffer. Isolated coronary vessels were cleaned and cut into rings of 2-3 mm in length as described (10). Once a stable contraction was obtained, acetylcholine (ACh), an endothelium-dependent vasodilator, was added to the bath in cumulative concentrations at 0.1, 1, 10, and 100 mM. After the response stabilized, the rings were washed and allowed to equilibrate to baseline once again. The procedure was repeated with another endothelium-dependent vasodilator, A-23187 (1, 10, 100, and 1000 nM), and then again with an endothelium-independent vasodilator, acidified NaNO2 (0.1, 1, 10, and 100 μM), at pH 2.

**Determination of Tissue Myeloperoxidase (MPO).** Myocardial activity of MPO, an enzyme occurring virtually exclusively in neutrophils, was determined using the method of Bradley et al. (11) as modified by Mullane et al. (12). One unit of MPO is defined as that quantity of enzyme hydrolyzing 1 mmol of peroxide per min at 25°C. The assays were performed without knowledge of the group to which each cat belonged.

**Cat Polymorphonuclear Leukocyte (PMN) Isolation and Labeling.** Peripheral blood (20 ml) was collected from the femoral artery and anticoagulated with citrate/phosphate/dextrose solution (Sigma) (1:4:10 vol/vol, anticoagulant to whole blood). Thirty milliliters of 0.9% NaCl was infused to replace the blood volume and the casts were allowed to stabilize for 30 min. PMNs were isolated by a procedure modified from Lafrando and Olsen (13). PMNs were collected from the 62%-82% interface of the Percoll:platelet-poor plasma gradient and washed twice with phosphate-buffered saline before being assayed for viability using trypan blue exclusion. Nonnecrotic PMNs separated by this method were typically >95% pure and >95% viable, as reported (8). Isolated autologous PMNs were then labeled with Zynaxis PKH2 fluorescent dye according to the method of Yuan and Fleming (14). This labeling procedure yields cells possessing normal morphology and function (14).

**PMN Adherence to Ischemic-Reperfusion Coronary Artery Endothelium.** During this PMN isolation and labeling period, the casts were subjected to 30 min of ischemia and 270 min of reperfusion (three casts were TGF-β1 treated and three casts were unstimulated) and left circumflex (LCX) coronary segments were isolated and removed as described. The arteries were opened and placed into the cell culture dishes containing 3 ml of K–H buffer. Autologous labeled PMNs (400,000 PMNs per ml) were then added to the arteries and allowed to incubate for 20 min. The arteries were removed and PMN adherence to coronary artery endothelium was counted using fluorescence microscopy. The number of PMNs per mm² of the coronary endothelium was counted in duplicate.

In six additional casts, peripheral blood was collected from the femoral artery and anticoagulated with citrate/phosphate/dextrose solution and PMNs were isolated as noted. LAD and LCX coronary arteries were removed and cut into 2- to 3-mm segments and placed in cell culture dishes filled with 3 ml of K–H buffer. The coronary rings were incubated for 2 hr with either 100 units of interleukin 1β (IL-1β) per ml (Genentech) or 1000 units of tumor necrosis factor α (TNF-α) per ml (Genentech) with or without TGF-β1 (200 ng/ml). After this 2-hr incubation period, 400,000 labeled neutrophils per ml were added to the cell culture dish. The coronary rings were removed 20 min later for fluorescence microscopy. In a second set of studies, labeled PMNs were incubated with TGF-β1 (200 ng/ml) for 30 min and subsequently added to the cell culture dish containing unstimulated coronary arteries. Then, leukotriene B4 (LTB4; 100 nM) or its vehicle was added to the dishes and the arteries were removed 20 min later and PMN adherence was counted.

**Statistical Analysis.** All values are presented as means ± standard error of the means of n independent experiments. All data were subjected to analysis of variance followed by the Bonferroni correction for post-hoc t test. Probabilities ≤ 0.05 were considered to be statistically significant.

**RESULTS**

**Cardiac Electrophysiologic and Hemodynamic Changes.** In the sham MI and reperfusion casts, we observed that i.v. administration of TGF-β1 (20 μg/kg) had no effect on any of the measured hemodynamic, electrocardiographic, or biochemical variables. In the two groups of MI + R cats, there were no significant differences in any of the variables observed before coronary occlusion. However, a few minutes after LAD occlusion, the ST segment of the ECG became significantly elevated, peaking at 20–40 min after coronary occlusion. After reperfusion, the ST segment decreased to nearly control values, indicating that reperfusion occurred. There was no significant difference in peak ST segment elevation between the two MI + R groups (0.21 ± 0.04 mV vs. 0.23 ± 0.05 mV), indicating that the ischemic insult was similar in these two MI + R groups. In both groups of MI + R cats, PRI decreased significantly after coronary occlusion and gradually returned to nearly control values after reperfusion. There were no significant differences between the two MI + R groups at any of the PRI readings, suggesting TGF-β1 did not appear to exert a hemodynamic mechanism of cardioprotection.

**Effect of TGF-β1 on Reperfusion Myocardial Injury.** To ascertain the effects of TGF-β1 on the degree of actual myocardial salvage of ischemic or necrotic tissue following reperfusion, we used an anatomic estimation of necrotic tissue to ischemia as an index of the extent of myocardial preservation. The weights of the areas of myocardium subjected to ischemia (area-at-risk) expressed as a percentage of the total left ventricular weight were not significantly different between the two MI + R groups (Fig. 1), indicating that the severity of the ischemic insult was comparable in both MI groups. In contrast, the mass of the necrotic myocardial tissue expressed as a percentage of the mass of the myocardial area-at-risk was not equivalent between the groups. About 30% of the jeopardized myocardium developed into necrotic tissue in the MI + vehicle group, whereas the TGF-β1-treated MI developed a significantly lower percentage of necrotic tissue than the MI + vehicle group (P < 0.01). These results were comparable whether the necrotic area was expressed as a percentage of area-at-risk or as a percentage of the total left ventricle (Fig. 1). Thus, the TGF-β1 treatment resulted in a significant attenuation in necrotic injury in the MI + R casts. Effect of TGF-β1 on Endothelial Dysfunction. Since endothelial dysfunction is an early and critical event in reperfusion injury, we also tested endothelial dysfunction by comparing vasoactivity of isolated coronary artery rings to the endothelium-dependent vasodilators ACh and A-23187 and to the endothelium-independent vasodilator NaNO2. Fig. 2 summarizes the vasorelaxant responses to ACh, A-23187, and NaNO2 in isolated cat coronary artery rings. Endothelium-dependent relaxation of ischemic LAD coronary rings to ACh...
and A-23187 was significantly greater in TGF-β1-treated MI + R cats compared to vehicle-treated cats. Thus, TGF-β1 significantly protected against the loss of endothelium-dependent relaxation observed in coronary artery rings isolated from cats subjected to MI and reperfusion. Nonischemic reperfused LCX coronary rings isolated from all groups relaxed fully to either the endothelium-dependent vasodilators (ACh and A-23187) or endothelium-independent vasodilator (NaNO2).

**Prevention of PMN Accumulation in Ischemic Myocardial Tissue by TGF-β1.** We measured MPO activity of the three different portions of the myocardium as a marker for neutrophil accumulation in ischemic tissue. In the nonischemic myocardium (i.e., area-not-at-risk), MPO activity was very low in both MI groups and there was no significant difference between them, indicating that few neutrophils infiltrated in the nonischemic myocardium. However, MI cats receiving only the vehicle exhibited a marked increase in MPO activity in the ischemic region, with a 10- to 12-fold increase in MPO activity in the necrotic myocardium. In contrast, TGF-β1-treated ischemic cats exhibited a significantly lower MPO activity in ischemic nonnecrotic myocardial tissue and necrotic myocardial tissue (Fig. 3). These results indicate that adherence and accumulation of neutrophils to ischemia-reperfused myocardium was markedly inhibited by TGF-β1.

**In Vivo Administration of TGF-β1 Inhibited PMN Adherence to Ischemia-Reperfused coronary Endothelium ex Vivo.**

![Fig. 2. Summary of responses of ischemia-reperfused LAD rings and nonischemic LCX coronary rings to 100 nM ACh, 1 μM A-23187, and 100 μM NaNO2. □, Sham MI plus TGF-β1; ◯, MI plus vehicle; ●, MI plus TGF-β1. Bar heights are means; brackets indicate SEM for 8–10 rings.](image)

![Fig. 3. MPO activity in area-at-risk, necrotic area, and area-not-at-risk in units/100 mg of tissue wet weight for the two MI + R groups. □, MI plus vehicle; ●, MI plus TGF-β1. Heights of bars are means; brackets represent SEM for six or seven samples. NS, not significant.](image)

An initial step in neutrophil-mediated injury is the increased adhesion of neutrophils to the vascular endothelium. In this study, we observed the effects of TGF-β1 on the ability of normal unstimulated PMNs to adhere to ischemia-reperfused coronary endothelium. When PMNs were added alone (i.e., unstimulated PMNs) to nonischemic-reperfused control LCX coronary arteries and incubated for 20 min, few neutrophils adhered to the endothelial surface. When PMNs were added to the ischemia-reperfused LAD coronary arteries isolated from the cats receiving only vehicle and incubated for 20 min, a dramatic increase in PMN adherence was observed (Fig. 4). Moreover, most of the PMNs spread out their cell boundaries and aggregated PMNs were often observed. In some cases, they formed clusters of cells with significant intercellular contact. In contrast, when PMNs were added to the ischemia-reperfused LAD coronary arteries isolated from the cats treated with TGF-β1, the number of PMNs adhering to the coronary endothelium was significantly inhibited (Fig. 4).

**In Vitro Addition of TGF-β1 on PMN Adherence to Coronary Endothelium.** Adherence of PMNs to endothelial cells may be increased by activation of either PMNs with chemotactic agents or endothelial cells with cytokines. To further clarify the mechanism by which TGF-β1 exerts its anti-PMN adherence effect, we compared the effects of TGF-β1 on LTB4-activated PMN adherence to normal unstimulated endothelium and unstimulated PMN adherence to cytokine-stimulated endothelium. Activation of PMNs with LTB4 significantly
increased PMN adherence to unstimulated endothelium. Addition of TGF-β1 (200 ng/ml) had no significant inhibitory effect on LTB4-activated PMN adherence to endothelium (Fig. 5). However, when TGF-β1 was added simultaneously with either TNF-α (1000 units/ml) or IL-1β (100 units/ml) and incubated for 2 hr, this cytokine-stimulated increase in PMN adherence was significantly attenuated (Fig. 6). Thus, TGF-β1 inhibits adherence of control PMNs to activated endothelial cells but does not inhibit adherence of activated PMNs to control endothelial cells.

**DISCUSSION**

Reperfusion of a previously ischemic vascular bed results in significant enhancement of tissue injury, the so-called "reperfusion injury" effect. TGF-β has been studied thus far in several ischemic-reperfusion states, including myocardial ischemia-reperfusion in the rat (6), splanchnic ischemia-reperfusion in the cat (7), and cerebral ischemia-reperfusion in the pig (15). In each case, TGF-β1 was the isoform of TGF-β studied. The common findings in all of these studies are that TGF-β1 exerted significant protective effects resulting in a smaller degree of tissue injury. Moreover, in all of these cases, evidence was presented that TGF-β1 preserved endothelial cell integrity. Endothelial cytoprotection thus becomes a critical mechanism for the salutary effects of TGF-β1.

**Fig. 4.** Effect of administration of TGF-β1 in vivo on unstimulated neutrophil adherence to nonischemic-reperfused LCX coronary endothelium and ischemic-reperfused LAD coronary endothelium in vitro. Data are expressed as numbers of PMNs per mm². MI, MI plus vehicle; MI, MI plus TGF-β1. Bar heights are means; brackets indicate ± SEM, and numbers at the bottom of the bars are numbers of coronary rings studied. NS, not significant.

**Fig. 5.** Effect of addition of TGF-β1 in vitro on unstimulated normal coronary artery endothelium. TGF-β1 was added simultaneously with LTB4 and incubated for 20 min. Data are expressed as numbers of PMNs per mm². Bar heights are means; brackets indicate ± SEM, and numbers at the bottom of the bars are numbers of coronary rings studied. NS, not significant.

**Fig. 6.** Effect of addition of TGF-β1 in vitro on unstimulated PMN adherence to cytokine-stimulated coronary artery endothelium. TGF-β1 was added simultaneously with either IL-1β or TNF-α and incubated for 2 hr. EC, endothelial cells. Data are expressed as numbers of PMNs per mm². Bar heights are means; brackets indicate ± SEM, and numbers at the bottom of the bars are numbers of coronary rings studied.

in reperfusion injury, as it is the earliest critical event in the reperfusion process (16, 17).

Another critical question to be answered is: Does TGF-β actually retard ischemic injury when indexed to the area-at-risk? In this feline model of ischemia-reperfusion, the degree of collateral flow is only 7% of the normal coronary blood flow and does not change significantly over the period of the experiment (7). In all previous studies employing TGF-β in ischemia-reperfusion, it was assumed that the entire organ or region was "at-risk." In the current study, we measured the exact area of myocardium-at-risk and indexed the degree of necrosis to this reference point, thus expressing myocardial damage as "% necrosis/area-at-risk."

Our results clearly show that TGF-β1, when administered 30 min prior to reperfusion at a dose of 20 μg/kg, markedly retards post-reperfusion cardiac necrosis (P < 0.01). This cardioprotective effect of TGF-β1 represents a necrosis/area-at-risk that is 60% lower than vehicle controls. This effect is clearly related to reperfusion injury rather than to direct ischemic injury since the TGF-β1 was absent for the first 60-min ischemic period. The dose of TGF-β1 employed in this study corresponds to a maximal circulating dose of 10 nm assuming all TGF-β1 is circulating and is not excreted or bound to plasma protein. Since the circulating TGF-β1 is on the order of 5 min (18) and since it avidly binds to α-secreted globulin (19), the true circulating concentration is probably <10 nm. Whatever the exact circulating concentration of TGF-β1, it failed to exert any significant systemic hemodynamic effect either on MABP, on HR, or on its product, the PRI. Therefore, the cardioprotective effect of TGF-β1 observed in this study was not due to an effect of TGF-β1 on myocardial oxygen demand. Previous studies on isolated cat coronary arteries have ruled out any direct coronary vasodilator effect (20), and studies on isolated cat cardiac muscle have ruled out any direct inotropic effects (20). Thus, it is unlikely that TGF-β1 protects the ischemic-reperfused myocardium via a hemodynamic effect.

A second major effect of TGF-β1 in our myocardial ischemia-reperfusion model was its endothelial preservation effect. TGF-β1 markedly preserved the ability of the ischemic-reperfused coronary vascular endothelium to release EDRF, now identified as nitric oxide (21, 22). This is consistent with the earlier reports that TGF-β1 preserves nitric oxide release in the rat coronary vascular endothelium (6) following ischemia-reperfusion as well as in the cat splanchnic vascular endothelium (7). Moreover, it helps explain the anti-TNF-α and anti-superoxide radical effects of TGF-β (6, 23) since TNF-α and superoxide radicals acutely oppose the actions of...
endothelium-derived nitric oxide (24, 25). The significance of preserving nitric oxide extends far beyond maintaining the ability of the vasculature to dilate, since endothelium-derived nitric oxide also elicits actions in platelets (26) and anti-adherence effects in neutrophils (27) and quenches superoxide radicals (28). Thus, maintaining nitric oxide at control levels on or near the endothelial surface promotes various anti-thrombotic and anti-inflammatory effects that could be of significant value in attenuating reperfusion injury. Therefore, TGF-β1 along with the stable pros-tacyclin analog taprostene (10), human recombinant superoxide dismutase (29), and a monoclonal antibody against the common β-chain of the neutrophil adhesive glycoprotein complex (16, 30) exhibit endothelial protection along with the preservation of reperfused myocardial tissue.

In the present study, we have also extended the data on endothelial integrity to include the role of neutrophils in myocardial reperfusion injury since PMNs have been shown to contribute to the post-reperfusion endothelial dysfunction (8). Toward this end, we determined cardiac MPO activity, an index of PMN accumulation into the post-reperfused myocardium, and the results indicate that ischemic-reperfused myocardial tissue (i.e., area-at-risk) as well as necrotic myocardial tissue exhibit a significant increase in cardiac MPO activity (Fig. 3). TGF-β1-treated ischemic-reperfused cats showed significantly lower MPO activity in both regions, indicating an anti-neutrophil effect of TGF-β1. To determine whether TGF-β1 exerted an anti-adherence effect, we examined the number of adherent PMNs to coronary endothelium at the end of the reperfusion period. As expected, there was a marked elevation in adherent PMNs in the ischemic-reperfused LAD coronary artery endothelium compared with the contralateral control LCX coronary artery in the same cat (Fig. 4). Moreover, TGF-β1-treated cats exhibited a markedly lower number of PMNs adhering to the reperfused LAD coronary vascular endothelium. This highly significant anti-adherent effect of TGF-β1 is consistent with earlier in vitro anti-leukocyte adherence reports of TGF-β1 by Gamble and Vadas (31) and confirmed by Bereta et al. (32) with tumor cell adherence to endothelial cells. This anti-adherence property of TGF-β1 appears to be a key mechanism of its amelioration of reperfusion injury.

We therefore raised the question whether TGF-β1 reduces PMN adherence to endothelial cells by inhibiting adherence molecules on the neutrophil or on the endothelial cell. In this connection, we activated isolated PMNs with the chemotactic eicosanoid LTβ2 to up-regulate the CD11/CD18 adherence complex (33) or to the platelet surface (34). TGF-β1 did not inhibit adherence of activated neutrophils to nonactivated coronary vascular cells. However, when we up-regulated the endothelial ligands for these adhesive proteins using an inflammatory promoting cytokine (e.g., TNF-α, IL-1β), TGF-β1 markedly inhibited adherence of nonactivated PMNs to the activated endothelium. These studies suggest endothelial mechanisms as the site of the TGF-β1 anti-PMN adherence effect. Although we have measured PMN adherence to the coronary artery endothelium, we recognize that the major site of PMN adherence is the venous endothelium. In dogs, we have established that PMNs bind to cardiac venous endothelium twice as avidly as to coronary artery endothelium (34). The relationships between artery and vein, however, appear to be similar. We have no direct information on which endothelial adhesive molecule(s) is the target (12) for the TGF-β1 adherence effects that occur in a cardiac non-candidate endothelial adherence molecules, including intercellular adhesive molecule 1 (ICAM-1) (35), endothelial leukocyte adhesion molecule 1 (now known as E-selectin) (36), and granular membrane protein 140 (now known as P-selectin) (37). Although prior studies indicate that a monoclonal antibody directed against ICAM-1 (38) protects the ischemic reperfused heart to a comparable degree as TGF-β1, we have no direct information on the other two adhesive molecules.

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