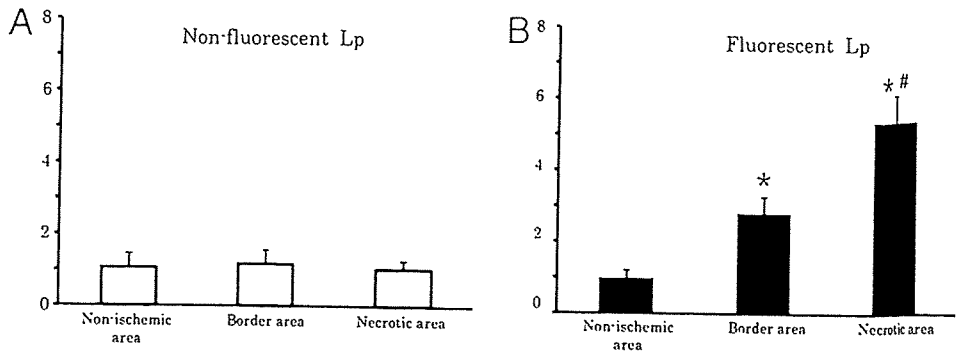

Figure 2 **Detection of Fluorescence-Labeled PEGylated Liposomes in Ischemic/Reperfused Myocardium**

Quantitative analysis of target-to-control fluorescent intensity ratio for each area in rats (n = 3 each group) that received nonfluorescent (A) or fluorescent (B) liposomes. The values of bioluminescence signals in the border and infarcted areas were expressed as the fold to that of the each nonischemic area. Values are expressed as the mean ± SEM (error bars). *p < 0.05 versus nonischemic areas. #p < 0.05 versus border areas.

somes. In contrast, the intravenous infusion of PEGylated liposomal adenosine at a dose of either 225 or 450 μg/kg/min did not significantly alter mean blood pressure (Fig. 4). Changes of the heart rate after infusion of PEGylated liposomal adenosine or free adenosine were similar to those observed for mean blood pressure (Fig. 4).

Effects of PEGylated liposomal adenosine on MI size. Baseline hemodynamic parameters were similar among all of the groups (Table 2). Intravenous infusion of free adenosine for 10 min reduced both the blood pressure and the heart rate, although these parameters returned to baseline within 5 min of ceasing infusion (Table 2). In contrast, hemodynamic parameters of the other groups were not altered (Table 2). The area at risk in the control group (61 ± 3%) did not differ compared with those of other groups that received liposomal adenosine. Intravenous infusion of PEGylated liposomal adenosine caused a dose-dependent decrease of MI size compared with that in the control group, whereas intravenous infusion of empty PEGylated liposomes or free adenosine did not (Fig. 5B).

The bolus injection of adenosine receptor antagonist did not alter the hemodynamic parameters (Table 3). The area at risk in the liposomal adenosine group (58 ± 3%) did not differ compared with those of other groups that received adenosine receptor antagonist. Infusion of 8-SPT, a nonspecific adenosine receptor antagonist, blunted the cardioprotective effect of liposomal adenosine (Fig. 6B). Furthermore, the infusion of the adenosine A_1, A_{2a}, A_{2b}, or A_3 receptor antagonist also blunted cardioprotective effects of liposomal adenosine (Fig. 6B). Infusion of 8-SPT alone did not significantly affect myocardial infarct size compared with the control (52 ± 5%, n = 4).

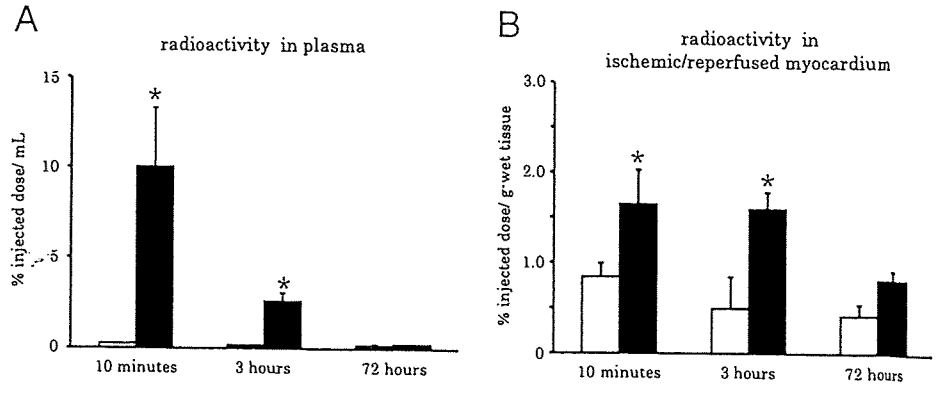

Figure 3 **Radioisotope-Labeled Adenosine in Plasma and Ischemic/Reperfused Myocardium**

(A) Changes in plasma radioactivity after infusion of radioisotope-labeled adenosine. Solid and open bars indicate the PEGylated liposomal adenosine and free adenosine groups, respectively (n = 4 each). In the PEGylated liposomal adenosine group, plasma radioactivity was markedly higher than in the free adenosine group. (B) Changes in radioactivity in ischemic/reperfused myocardium. Solid and open bars indicate the PEGylated liposomal adenosine and free adenosine groups, respectively (n = 4 each). In the PEGylated liposomal adenosine group, myocardial radioactivity was markedly higher than in the free adenosine group. Values are expressed as the mean ± SEM (error bars). *p < 0.05 versus the free adenosine group at the corresponding time.

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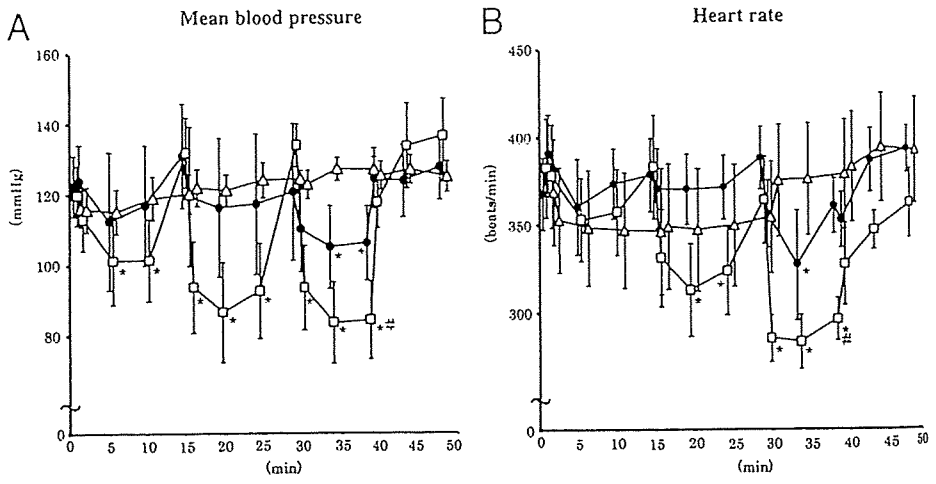

Figure 4 Hemodynamic Effects of PEGylated Liposomal Adenosine

Changes in the mean blood pressure (A) and heart rate (B) after intravenous infusion of various doses of empty PEGylated liposomes (triangles), PEGylated liposomal adenosine (circles), or free adenosine (squares) (n = 8 each). Values are expressed as the mean ± SEM. *p < 0.05 versus baseline at the corresponding group. #p < 0.05 versus PEGylated liposomes.

Discussion

In the present study, EM, bioluminescence ex vivo imaging, and fluorescent analysis revealed the accumulation of liposomes in the border (noninfarcted areas at risk) as well as infarcted ones, but not nonischemic myocardium, at 3 h after MI. These findings suggested that liposomes could specifically accumulate in ischemic/reperfused myocardium. Interestingly, EM revealed the existence of liposomes at sites where endothelial integrity was still morphologically maintained. Endothelial dysfunction such as enhanced permeability is induced by ischemic insult without morphological endothelial disruption (3,15). Enhanced permeability might lead to the accumulation of liposomes in the border as well as infarcted area, which will contribute to salvage the ischemic/reperfused myocardium. However, further investigation will be needed to determine the precise mechanism by which liposomes accumulate in ischemic/reperfused myocardium.

Analysis using RI-labeled adenosine encapsulated in liposomes revealed that plasma radioactivity was markedly higher in the PEGylated liposomal adenosine group compared with the free adenosine group. This indicates that encapsulation of adenosine by PEGylated liposomes considerably prolonged its residence time in the circulation and delayed its degradation. Consistent with the histological data, RI-labeled adenosine also showed preferential accumulation in ischemic/reperfused myocardium.

Table 2 Effects of Liposomal Adenosine on Hemodynamic Parameters

		Ischemia				Reperfusion	
	Baseline	0 min	15 min	25 min	30 min	5 min	10 min
Mean blood pressure (mm Hg)							
Saline	122 ± 5	102 ± 10	108 ± 7	107 ± 9	108 ± 7	105 ± 9	104 ± 9
Vehicle	127 ± 4	109 ± 8	108 ± 7	111 ± 9	111 ± 5	105 ± 5	103 ± 5
Free-Ado	124 ± 8	115 ± 8	111 ± 5	109 ± 4	66 ± 4*	62 ± 4*	112 ± 6
Lp-Ado 50 μg/kg/min	121 ± 5	106 ± 6	105 ± 6	110 ± 10	102 ± 6	101 ± 6	104 ± 4
Lp-Ado 150 μg/kg/min	122 ± 3	107 ± 6	107 ± 6	109 ± 11	105 ± 6	100 ± 6	103 ± 4
Lp-Ado 450 μg/kg/min	124 ± 3	104 ± 6	105 ± 6	107 ± 5	102 ± 6	99 ± 6	104 ± 4
Heart rate (beats/min)							
Saline	363 ± 22	366 ± 19	369 ± 14	413 ± 22	372 ± 12	372 ± 16	371 ± 14
Vehicle	363 ± 32	363 ± 6	383 ± 6	396 ± 25	367 ± 6	374 ± 7	372 ± 7
Free-Ado	360 ± 18	361 ± 17	384 ± 13	379 ± 18	305 ± 11*	293 ± 13*	356 ± 14
Lp-Ado 50 μg/kg/min	378 ± 19	386 ± 21	366 ± 12	376 ± 12	367 ± 19	369 ± 9	377 ± 17
Lp-Ado 150 μg/kg/min	388 ± 27	376 ± 20	371 ± 14	377 ± 13	378 ± 16	373 ± 16	369 ± 17
Lp-Ado 450 μg/kg/min	368 ± 17	376 ± 21	361 ± 13	386 ± 15	368 ± 15	363 ± 6	367 ± 7

Values are expressed as mean ± SEM. *p < 0.05 versus baseline.
Free-Ado = free adenosine; Lp-Ado = PEGylated liposomal adenosine; PEG = polyethylene glycol; vehicle = PEGylated liposomes.

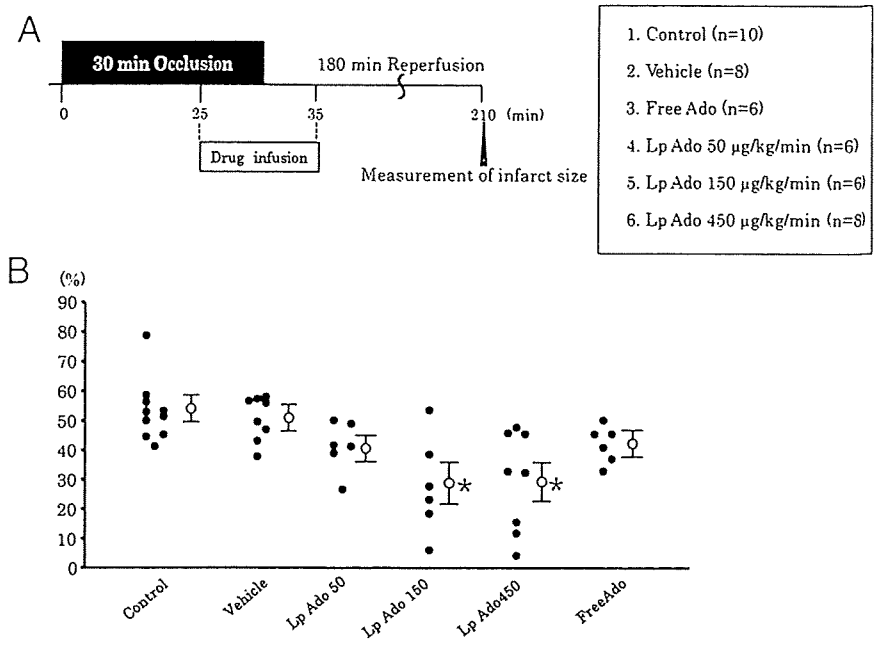

Figure 5 Dose-Dependent Effects of PEGylated Liposomal Adenosine on Myocardial Infarct Size

(A) Protocol showing the timing of infusion of PEGylated liposomal adenosine and of infarct size measurement. (B) Myocardial infarct size. Values are expressed as mean ± SEM (error bars). *p < 0.05 versus the control group. Free-Ado = free adenosine; Lp-Ado = PEGylated liposomal adenosine; Vehicle = PEGylated liposomes.

Furthermore, this study showed that PEGylated liposomal adenosine had a weaker effect on the blood pressure and heart rate than free adenosine. Thus, encapsulating adenosine in PEGylated liposomes attenuated its vasodilatory and negative chronotropic effects, presumably by reducing the amount of circulating free adenosine. However, the changes of hemodynamic parameters in this in vivo model suggested that significant release of adenosine from PEGylated liposomes would still occur if a large dose of liposomal adenosine (e.g., 900 μg/kg/min) were administered. Thus, further investigation of the in vivo pharmacodynamics of PEGylated liposomal adenosine is needed.

An intravenous infusion of PEGylated liposomal adenosine at the maximum dose that did not disturb hemodynamic parameters for 10 min before reperfusion reduced MI size in a dose-dependent manner, and this improvement was blocked by 8-SPT, a nonselective adenosine receptor antagonist. These findings suggest that adenosine released from liposomes acts via an adenosine receptor-dependent pathway. One possible mechanism by which PEGylated lipo-

Table 3 Effects of Adenosine Receptor Antagonist on Hemodynamic Parameters

		Ischemia				Reperfusion	
	Baseline	0 min	15 min	25 min	30 min	5 min	10 min
Mean blood pressure (mm Hg)							
Lp-Ado + 8SPT	120 ± 6	113 ± 4	112 ± 6	112 ± 5	107 ± 6	102 ± 8	109 ± 7
Lp-Ado + DPCPX	130 ± 6	105 ± 4	121 ± 4	100 ± 10	122 ± 6	120 ± 6	111 ± 4
Lp-Ado + SCH58261	132 ± 2	98 ± 12	99 ± 8	110 ± 8	118 ± 10	113 ± 10	109 ± 6
Lp-Ado + MRS1754	130 ± 3	95 ± 12	106 ± 8	105 ± 10	100 ± 10	96 ± 10	99 ± 7
Lp-Ado + MRS1523	130 ± 2	109 ± 8	104 ± 8	105 ± 9	100 ± 9	101 ± 10	104 ± 6
Heart rate (beats/min)							
Lp-Ado + 8SPT	404 ± 17	385 ± 10	374 ± 8	396 ± 8	389 ± 9	383 ± 8	385 ± 9
Lp-Ado + DPCPX	396 ± 24	380 ± 11	399 ± 9	398 ± 12	385 ± 9	382 ± 9	380 ± 7
Lp-Ado + SCH58261	393 ± 14	399 ± 15	381 ± 9	395 ± 15	376 ± 9	373 ± 9	385 ± 7
Lp-Ado + MRS1754	398 ± 14	392 ± 11	401 ± 9	379 ± 15	378 ± 9	374 ± 9	377 ± 7
Lp-Ado + MRS1523	396 ± 9	390 ± 11	390 ± 11	392 ± 10	373 ± 9	391 ± 7	388 ± 11

Values were expressed as mean ± SEM. *p < 0.05 versus baseline.
Lp-Ado = PEGylated liposomal adenosine; PEG = polyethylene glycol; Vehicle = PEGylated liposomes.

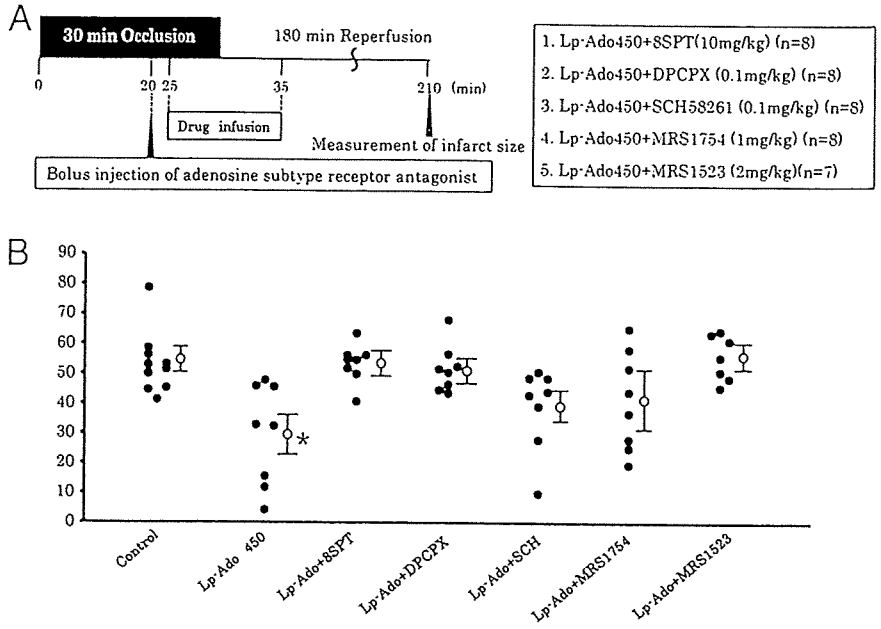

Figure 6 **Effects of Adenosine Receptor Antagonists on Myocardial Infarct Size**

(A) Protocol showing the timing of infusion of PEGylated liposomal adenosine and bolus injection adenosine receptor antagonists. (B) Myocardial infarct size. Values are expressed as the mean ± SEM (error bars). *$p < 0.05$ versus the control group. The abbreviations for adenosine antagonists were described in the text. Abbreviations as in Figure 5.

somes could augment cardioprotective effects of liposomal adenosine with minimum effects on hemodynamic parameters is the enhanced accumulation of PEGylated liposomal adenosine in ischemic/reperfused myocardium, which could augment various beneficial actions such as preventing calcium overload in the myocardium (5). The prolonged persistence of PEGylated liposomal adenosine would also increase its beneficial effect on ischemic/reperfused myocardium. Although continuous high-dose, long-term infusion of free adenosine was reported to reduce infarct size in rats (16), the present study did not confirm such a cardioprotective effect, probably because the total dose of free adenosine that we used was not high enough.

We found that myocardial infarct size in the group that received PEGylated liposomal adenosine with the antagonist of adenosine A_1, A_{2a}, A_{2b}, or A_3 subtype receptor was no different from the control group, indicating that every adenosine subtype receptor could possibly play a role in mediating cardioprotection by liposomal adenosine and that it was difficult to identify one particular subtype in the present study. Numerous studies reported that A_1, A_{2a}, A_{2b}, and A_3 receptors have been involved in cardioprotection against ischemia/reperfusion injury, and it remains controversial which adenosine subtype receptor is most responsible for cardioprotection (17–20). Furthermore, because the adenosine receptor antagonists used in the present study had some nonspecific effects, future investigation will be needed to examine the precise role of each adenosine receptor subtype using genetically engineered mice.

Because liposomal adenosine infused during reperfusion could reduce MI size, this agent could be a candidate for the adjunctive therapy of patients with acute MI. Importantly, adenosine is currently used for the diagnosis of ischemic heart disease and PEGylated liposomes are used to deliver anticancer agents (21). Thus, it should not be difficult to introduce PEGylated liposomal adenosine into clinical practice. Finally, PEGylated liposomes may provide a useful drug delivery system for targeting ischemic/reperfused myocardium with other agents.

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REFERENCES

1. Papahadjopoulos D, Allen TM, Gabizon A, et al. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc Natl Acad Sci U S A 1991;24:11460–4.

2. Horwitz LD, Kaufman D, Keller MW, Kong Y. Time course of coronary endothelial healing after injury due to ischemia and reperfusion. Circulation 1994;90:2439–47.
3. Dauber IM, Van Benthuysen KM, McMurtry IF, et al. Functional coronary microvascular injury evident as increased permeability due to brief ischemia and reperfusion. Circ Res 1990;66:986–98.
4. Forman MB, Stone GW, Jackson EK. Role of adenosine as adjunctive therapy in acute myocardial infarction. Cardiovasc Drug Rev 2006;24:116–47.
5. Mubagwa K, Flameng W. Adenosine, adenosine receptors and myocardial protection: an updated overview. Cardiovasc Res 2001;52:25–39.
6. Mahaffey KW, Pume JA, Barbagelata NA, et al. Adenosine as an adjunct to thrombolytic therapy for acute myocardial infarction: results of a multicenter, randomized, placebo-controlled trial: the Acute Myocardial Infarction STudy of ADenosine (AMISTAD) trial. J Am Coll Cardiol 1999;34:1711–20.
7. Ross AM, Gibbons RJ, Stone GW, Kloner RA, Alexander RW, for the AMISTAD-II ÅInvestigators. A randomized, double-blinded, placebo-controlled multicenter trial of adenosine as an adjunct to reperfusion in the treatment of acute myocardial infarction (AMISTAD-II). J Am Coll Cardiol 2005;45:1775–80.
8. Bullard AJ, Govewalla P, Yellon DM. Erythropoietin protects the myocardium against reperfusion injury in vitro and in vivo. Basic Res Cardiol 2005;100:397–403.
9. Hannon JP, Tigani B, Wolber C, et al. Evidence for an atypical receptor mediating the augmented bronchoconstrictor response to adenosine induced by allergen challenge in activity sensitized Brown Norway rats. Br J Pharmacol 2002;135:685–96.
10. Kin H, Zatta AJ, Lofye MT, et al. Postconditioning reduces infarct size via adenosine receptor activation by endogenous adenosine. Cardiovasc Res 2005;67:124–33.
11. Hinschen AK, RoseMeyer RB, Headrick JP. Adenosine receptor subtypes mediating coronary vasodilation in rat hearts. J Cardiovasc Pharmacol 2003;41:73–80.
12. Kaeffeer N, Richard V, Francois A, Lallemand F, Henry JP, Thuillez C. Preconditioning prevents chronic reperfusion-induced coronary endothelial dysfunction in rats. Am J Physiol 1996;271:H842–9.
13. M Shimizu, Miwa K, Hashimoto Y, Goto A. Encapsulating of chicken egg yolk immunoglobulin G (IgY) by liposomes. Biosci Biotechnol Biochem 1993;57:1445–9.
14. Kasuya T, Jung J, Kadoya H, et al. In vivo delivery of bionanocapsules displaying phaseolus vulgaris agglutinin-L(4) isolectin to malignant tumors overexpressing N-acetylglucosaminlyltransferase V. Hum Gene Ther 2008;19:887–95.
15. Kim YD, Fomsgaard JS, Heim KF, et al. Brief ischemia-reperfusion induces stunning of endothelium in canine coronary artery. Circulation 1992;85:1473–82.
16. Canyon SJ, Dobson GP. Protection against ventricular arrhythmias and cardiac death using adenosine and lidocaine during regional ischemia in the in vivo rat. Am J Physiol Heart Circ Physiol 2004;287:H1286–95.
17. Yaar R, Jones MR, Chen JF, Ravid K. Animal models for the study of adenosineÅreceptor function. J Cell Physiol 2005;202:9–20.
18. Norton ED, Jackson EK, Turner MB, Virmani R, Forman MB. The effects of intravenous infusions of selective adenosine A_1-receptor and A_2-receptor agonists on myocardial reperfusion injury. Am Heart J 1992;123:332–8.
19. Xu Z, Mueller RA, Park SS, Boysen PG, Cohen MV, Downey JM. Cardioprotection with adenosine A_2 receptor activation at reperfusion. J Cardiovasc Pharmacol 2005;46:794–802.
20. Vinten-Johansen J. Postconditioning: a mechanical maneuver that triggers biological and molecular cardioprotective responses to reperfusion. Heart Fail Rev 2007;12:235–344.
21. Lasic DD. Doxorubicin in sterically stabilized liposomes. Nature 1996;380:561–2.

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