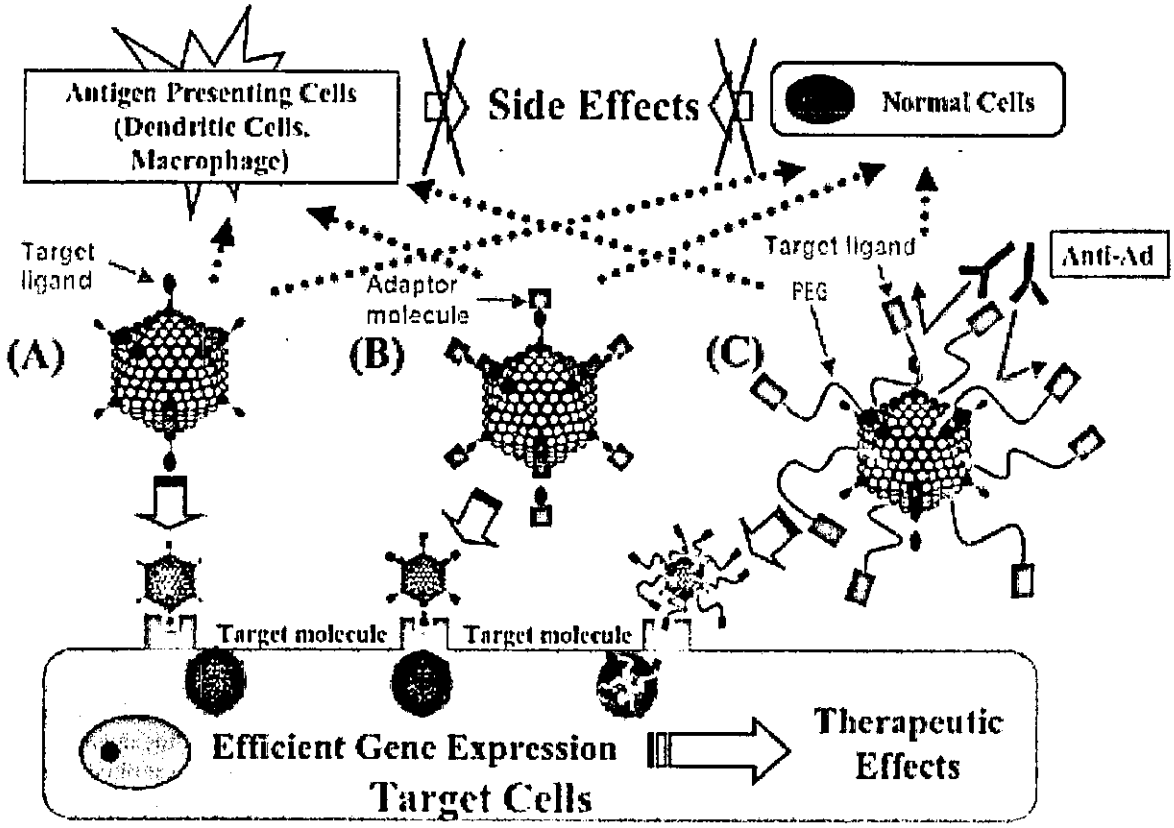


FIG. 1. Three approaches to developing targeted Ad vectors. (A) Genetic modification of virus capsid. (B) Modification by the use of adaptor molecules. (C) Chemical modification by polymers with ligands.

## KINETICS OF ADENOVIRUS VECTOR-MEDIATED GENE TRANSFER *IN VIVO*

Important determinants of virus clearance from the bloodstream include interactions between viral components and cellular receptors, virion size, net charge of the viral particle, and anatomical barriers, such as tightness of the basal membrane of endothelial cells. Understanding factors that impact on the kinetics of blood clearance and the biodistribution of Ad vectors would be beneficial to advancing their application as therapeutic agents.

Systemically administered Ad vectors are rapidly cleared from the blood of mice, with a half-life of less than 3 min (Alemany *et al.*, 2000; Koizumi *et al.*, 2003a; Sakurai *et al.*, 2003b). Liver Kupffer cells play a central role in clearing Ad genomes from the bloodstream (Lieber *et al.*, 1997; Wolff *et al.*, 1997; Worgall *et al.*, 1997). Activated Kupffer cells (and monocytes and resident macrophages) release proinflammatory cytokines/chemokines such as interleukin 6 (IL-6), tumor necrosis factor $\alpha$ (TNF-$\alpha$), interferon $\gamma$-inducible protein 10 (IP-10), and RANTES (regulated on activation, normal T cell expressed and secreted), causing the activation of an innate immune response (Liu and Muruve, 2003). It has been proposed that a low dose of Ad vectors ($\sim 10^{10}$ vector particles) is rapidly sequestered by Kupffer cells (nonparenchymal cells), whereas higher doses of Ad vectors are delivered into both Kupffer cells and parenchymal cells, leading to a nonlinear dose response in hepatic transgene expression (Tao *et al.*, 2001). At a dose of $3.0 \times 10^{10}$ vector particles, Ad vectors are likely to be equally distributed to Kupffer and parenchymal cells (Koizumi *et al.*, 2003a).

The liver directivity of the systematically administered Ad vectors can also be applied when local administration of the vectors is performed. Even if the Ad vector is injected into local tissues such as tumors, large amounts of vector are distributed into the bloodstream and targeted into the liver, causing unwanted side effects (Mizuguchi and Hayakawa, 2002b; Okada *et al.*, 2003). The process of Ad vector-mediated liver transduction is influenced by interactions between viral components and cellular receptors (discussed in Truly Targeted Adenovirus Vectors, below), the size of the sinusoidal fenestrae (Fechner *et al.*, 1999; Lievens *et al.*, 2004), and the complement system (Zinn *et al.*, 2004). Lievens *et al.* showed that Ad vector-mediated liver transduction in Dutch Belt rabbits, with 124-nm sinusoidal fenestrae, is significantly higher than that in New Zealand White rabbits, which have 108-nm sinusoidal fenestrae, and Fauve de Bourgogne rabbits with 105-nm sinusoidal fenestrae (Lievens *et al.*, 2004). The increase in sinusoidal fenestrae to 123 nm in New Zealand White rabbits by the intraportal injection of sodium decanoate enhances Ad vector-mediated liver transduction, confirming that the size of the sinusoidal

fenestrae is an important determinant for liver transduction (Lievens *et al.*, 2004). For targeting Ad vector to extrahepatic tissues, it is important to avoid distribution into parenchymal and nonparenchymal (Kupffer) cells of the liver as well as other tissues, such as spleen.

## APPROACHES TO DEVELOPING TARGETED ADENOVIRUS VECTORS

### *Genetic modification of the virus capsid*

*Modification of virus tropism.* Modification of the fiber proteins has been used to successfully overcome barriers to transduction due to a paucity of CAR. Two approaches have been used for this purpose. One is the addition of foreign peptides to the HI loop or C terminus of the fiber knob (Wickham *et al.*, 1997; Dmitriev *et al.*, 1998; Krasnykh *et al.*, 1998; Mizuguchi *et al.*, 2001; Koizumi *et al.*, 2003b). Another is the substitution of fibers derived from other Ad serotypes, which bind to receptor molecules other than CAR (Gall *et al.*, 1996; Stevenson *et al.*, 1997; Chillon *et al.*, 1999; Shayakhmetov *et al.*, 2000; Mizuguchi and Hayakawa, 2002a). Both approaches allow Ad tropism to be expanded (or changed) via binding of the modified fiber protein with a different cellular receptor.

Expanded and higher rates of gene transfer have been reported on the basis of the use of mutant fiber proteins containing an Arg-Gly-Asp (RGD) peptide (Wickham *et al.*, 1997; Dmitriev *et al.*, 1998; Hidaka *et al.*, 1999; Mizuguchi *et al.*, 2001) or a stretch of lysine residues (KKKKKKK [$K_7$] peptide) (Wickham *et al.*, 1997; Hidaka *et al.*, 1999), which target $\alpha_v$ integrins or heparan sulfates to the cellular surface, respectively. The RGD peptide has been displayed in the HI loop or C terminus of the fiber knob, whereas the $K_7$ peptide has been displayed at the C terminus of the fiber knob. There have also been reports of inserting the peptides into the HI loop of the fiber knob, including those discovered by phage display library to show high affinity for vascular endothelial cells (Nicklin *et al.*, 2000), cancer cells (Nicklin *et al.*, 2003), transferrin receptor (Xia *et al.*, 2000), and vascular smooth muscle cells (Work *et al.*, 2004).

Altered vector tropism was reported by substitution of the Ad type 5 (Ad5) fiber protein into that of Ad3, Ad7, Ad11, Ad16, Ad17, Ad35, and others (Gall *et al.*, 1996; Stevenson *et al.*, 1997; Chillon *et al.*, 1999; Shayakhmetov *et al.*, 2000; Goossens *et al.*, 2001; Havenga *et al.*, 2001; Rea *et al.*, 2001; Stecher *et al.*, 2001; Mizuguchi and Hayakawa, 2002a). Most Ad serotypes belonging to the subgroups A, C, D, E, and F use CAR as the initial receptor for the virion (Roelvink *et al.*, 1998), whereas Ad serotype B uses other molecules for infection (Roelvink *et al.*, 1998; Arnberg *et al.*, 2000a,b; Law and Davidson, 2002; Burmeister *et al.*, 2004). Ad8, Ad19, and Ad37, which belong to serotype D, use sialic acids as the primary receptor (Arnberg *et al.*, 2000a,b; Burmeister *et al.*, 2004). CD46, CD80, and CD86 were identified as cellular receptor(s) of Ad belonging to subgroup B, including Ad3, Ad11, Ad14, Ad16, Ad21, Ad35, and Ad50 (Gaggar *et al.*, 2003; Segerman *et al.*, 2003; Short *et al.*, 2004). Human CD34-positive cells, dendritic cells, synoviocytes, vascular endothelial cells (ECs), and smooth muscle cells (SMCs), which were poorly transfectable by conventional Ad vectors, were efficiently transfected by fiber-substituted Ad vectors (Shayakhmetov *et al.*, 2000; Goossens *et al.*, 2001; Havenga *et al.*, 2001; Okada *et al.*, 2001; Rea *et al.*, 2001). Mercier *et al.* described the creation of a chimeric Ad vector encoding the reovirus attachment protein $\sigma$1, which targets cells expressing the junctional adhesion molecule 1 (JAM1) (Mercier *et al.*, 2004).

When modified Ad vectors are injected locally into target tissue expressing corresponding receptors, the affinity of the vector for the cells increases, thereby resulting not only in higher transduction efficiency, but also in decreased vector dissemination. We reported that the intratumoral administration of luciferase-expressing Ad vectors containing the RGD peptide in the HI loop of the fiber knob resulted in nearly 40 times more transgene production in tumor, but 8 times less transgene expression in liver in the B16 mouse melanoma model as compared with conventional Ad vectors (Mizuguchi and Hayakawa, 2002b).

Other candidate locations for insertion of foreign ligands into the Ad capsid are the pIX, the penton base, and the hypervariable region (HVR) 5 of hexon loop L1 (Wickham *et al.*, 1995; Vigne *et al.*, 1999; Dmitriev *et al.*, 2002; Vellinga *et al.*, 2004). Among them, pIX seems to be the most promising. pIX is a minor structural protein that is contained in the Ad virion, and enhances the structural integrity of the particles by stabilizing hexon–hexon interaction (Ghosh-Choudhury *et al.*, 1987; Furcinitti *et al.*, 1989). It also plays a role in transcriptional activity and nuclear reorganization (Rosa-Calatrava *et al.*, 2001). Foreign ligands are displayed at the C terminus of the pIX of Ad (Dmitriev *et al.*, 2002). The attractive characteristics of ligand insertion into the pIX region is that the C terminus of pIX tolerates the insertion of large peptides. By incorporation of the pIX–green fluorescent protein (GFP) fusion protein, a fluorescent Ad was generated (Le *et al.*, 2004; Meulenbroek *et al.*, 2004). The insertion of higher affinity ligands such as single-chain antibodies (scFv) would be ideal, although generating such Ad vectors might be difficult because of impaired assembly of complex scFv–pIX fusion proteins in the nucleus. One problem with pIX fusions is that Ad pIX resides below the top of the hexon capsomer, within the core of the virus. This problem was circumvented by incorporating an $\alpha$-helical spacer into the ligand–pIX fusion protein so as to lift the ligand and expose it to the surface of the capsid (Vellinga *et al.*, 2004). However, Ad vectors containing the RGD motif in the C terminus of pIX with $\alpha$-helical spacers are likely to be less efficient than Ad vectors containing the RGD motif in the HI loop of the fiber knob (Vellinga *et al.*, 2004). Additional modification may be required for improved efficacy and specificity of retargeting.

Several groups have developed an Ad vector from an entire Ad35, and have demonstrated higher transduction efficiency for the Ad35 vector into human CD34-positive cells and dendritic cells compared with the conventional Ad5 vector (Gao *et al.*, 2003; Sakurai *et al.*, 2003a,b; Seshidhar Reddy *et al.*, 2003; Vogels *et al.*, 2003). In addition, Ad35 vectors have the advantage of evading humoral immune responses against Ad5. However, fiber-substituted Ad5 vectors containing fiber proteins of another serotype do not circumvent the immune response against Ad5 (Gall *et al.*, 1996; Ophorst *et al.*, 2004), because hexon is the major target of host-neutralizing antibodies in Ad5 infec-

tion (Gall *et al.*, 1996, 1998; Roy *et al.*, 1998). The Ad35 vector would be an effective alternative for use in persons with neutralizing antibodies in Ad5, and in the second injection when the Ad5 vector is used in the first injection of *in vivo* gene therapy.

*Truly targeted adenovirus vectors.* Although modifications described above yield Ad vectors with greatly improved transduction to many cells lacking in CAR expression, when systemically administered, vector dissemination, resulting in accumulation in liver, is unavoidable. To create a strictly targeted Ad vector, two basic requirements must be met. The first is construction of vectors that abolish natural viral tropism. The second is identification and incorporation of a foreign ligand with high affinity for a specific cellular receptor into the capsid of Ad vectors.

The capsid proteins determine the tropism of Ad. Because the fiber knob binds with CAR, this interaction first must be abolished. Mutation of the AB, DE, or FG loop of the fiber knob has been reported to abolish the fiber–CAR interaction (Bewley *et al.*, 1999; Kirby *et al.*, 1999; Roelvink *et al.*, 1999). These mutations of the fiber knob greatly reduce the transduction efficiency of Ad vectors to CAR-positive cells *in vitro*. In another strategy, Nakamura *et al.* replaced the tail, shaft, and knob domains of the Ad5 fiber with those of the Ad40 short fiber, which is hypothesized not to bind to any receptors (Nakamura *et al.*, 2003). In addition, interaction of the RGD motif of the penton base with $\alpha_v$ integrin must be abolished, although this interaction might be minor, at least *in vitro* (Mizuguchi *et al.*, 2002). The ablation of $\alpha_v$ integrin binding was accomplished by deletion of the RGD motif of the penton bases. Several articles reported that a single mutation of either the fiber knob or penton base does not change the biodistribution of Ad vectors in mice after *in vivo* injection (Alemany and Curiel, 2001; Leissner *et al.*, 2001; Mizuguchi *et al.*, 2002), whereas double mutation reduces liver transduction (Einfeld *et al.*, 2001; Koizumi *et al.*, 2003a), although two groups showed that double mutation also does not reduce liver transduction (Martin *et al.*, 2003; Smith *et al.*, 2003b). The reason for this discrepancy is unclear. However, Nicol *et al.* reported that combining fiber knob and penton base mutations reduces liver transduction by 509-fold in rats, an effect not observed in parallel experiments in mice (Nicol *et al.*, 2004). Subtle differences among the vectors, such as differences in mutated amino acids, experimental animal strains used, or injected doses, might have caused these discrepancies. Furthermore, the fiber shaft domain of Ad5 was reported to be involved in accumulation in the mouse liver of systemically administrated Ad vectors (Nakamura *et al.*, 2003; Smith *et al.*, 2003b), possibly because of the interaction of the KKTK (Lys-Lys-Thr-Lys) motif on the fiber shaft of Ad5 with heparan sulfate (Smith *et al.*, 2003b). This effect was also observed in nonhuman primate (cynomolgus monkey) models (Smith *et al.*, 2003a). According to our data, triple mutation of

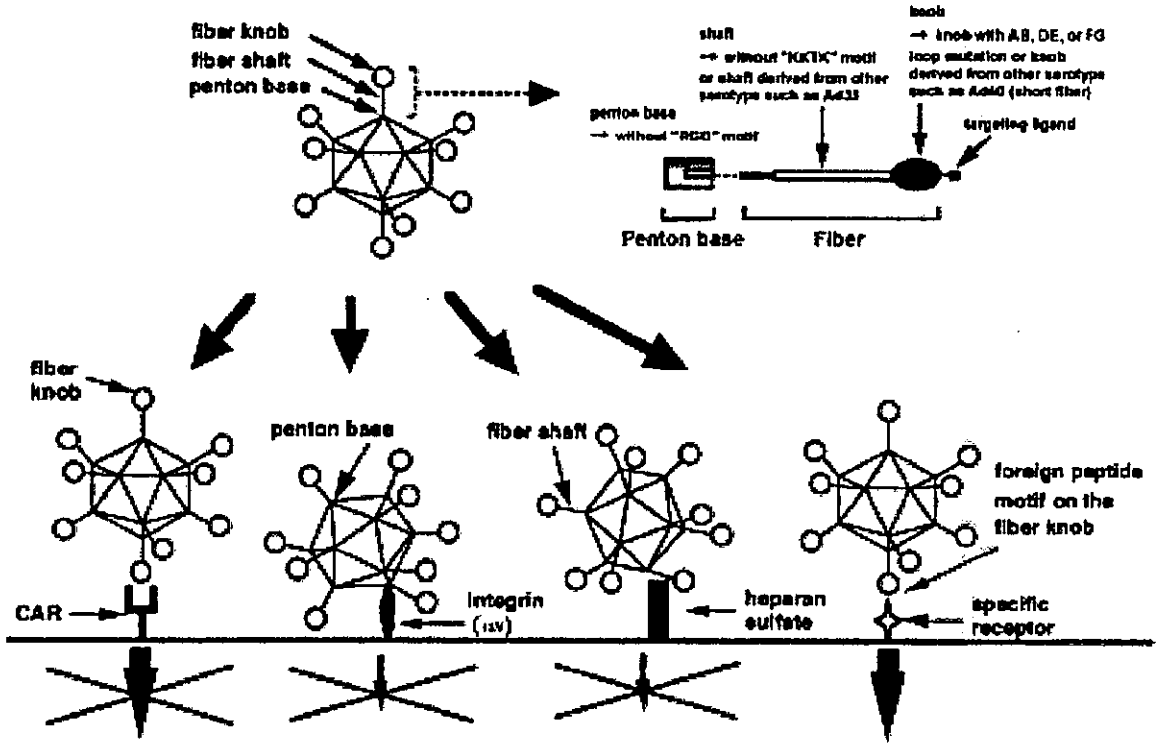


FIG. 2. Schematic diagram of Ad vectors targeted by the genetic approach. The CAR-, $\alpha_v$ integrin-, and heparan sulfate-binding activities of the Ad capsid are completely ablated by mutations in the fiber knob, the fiber shaft, and the penton base, respectively (Koizumi *et al.*, 2003a; Nicol *et al.*, 2004). Targeting ligands should be incorporated in the virus capsid, such as the fiber, the penton base, the hexon, or pIX. The resulting targeted Ad vectors transduce cells via the incorporated foreign ligand-dependent, CAR-, $\alpha_v$ integrin-, and heparan sulfate-independent pathway.

the fiber knob, shaft, and penton base mediated levels of liver transduction more than 30,000-fold lower than that of conventional Ad vectors (Koizumi *et al.*, 2003a). This vector contains a CAR-binding ablated mutant fiber knob derived from Ad5, a fiber shaft derived from Ad35 (the fiber shaft of Ad35 does not have the KKTK motif and is shorter than that of Ad5 [Ad5 fiber shaft, 6 $\beta$ repeats; Ad5 fiber shaft, 22 $\beta$ repeats]), a fiber tail derived from Ad5, and a mutant penton base of Ad5 without the RGD motif. Ad vectors, with mutations in two domains of the fiber knob, the fiber shaft, and the penton base, showed a level of liver transduction intermediate between that of conventional Ad vectors and the triple-mutant Ad vectors (Koizumi *et al.*, 2003a). Nicol *et al.* reached a similar conclusion in a rat model (Nicol *et al.*, 2004). Thus, Ad tropism would be determined by at least three factors: the fiber knob, the fiber shaft, and the RGD motif of the penton base (Figs. 1A and 2). Triple mutations, including the fiber knob, the fiber shaft, and the RGD motif of the penton base, should be preferable for the platform of targeted Ad vectors.

A detailed study on vector distribution to the liver, however, suggested that triple-mutant Ad vectors distribute to nonparenchymal cells to a similar extent as conventional vectors, and that both vectors are cleared rapidly from the bloodstream, having a half-life of less than 2 min (Koizumi *et al.*, 2003a). This nonparenchymal cell-mediated clearance might present an obstacle to the development of targeted Ad vectors that incorporate a foreign ligand into the viral capsid. One promising strategy to overcome this problem might be intraperitoneal, not intravenous, injection of the vector. Akiyama *et al.* reported that the intraperitoneal administration of CAR and integrin binding-ablated Ad vectors increases their persistence in the bloodstream, although the mechanism by which this occurs is unknown (Akiyama *et al.*, 2004). Extended release of the vector from the cavity might change its pharmacokinetics. More detailed study is needed to clarify nonparenchymal cell-mediated vector clearance. Lower clearance from the bloodstream may lead to increased delivery of the vector to the tissue of interest, if an appropriate targeting ligand is incorporated into the vector.

The identification of targeting ligands that are displayed in the capsid, such as fiber and pIX, is another challenge. A display library using filamentous phage is widely used for the identification of functional peptides for targeting. Although some success in identifying peptide ligands for the targeted Ad vectors was reported (Nicklin *et al.*, 2000; Xia *et al.*, 2000; Work *et al.*, 2004), most peptides that are identified by phage display libraries are not functional when they are displayed in the fiber knob of Ad vectors. Foreign peptides inserted into the HI loop of the fiber knob are constrained at both the N and C termini, whereas peptides inserted at the C terminus of the fiber knob are constrained only at the N terminus. In contrast, peptides identified by filamentous phage display library are constrained only at the C terminus, when the peptides are displayed as a fusion protein with the product of gene III of the phage. The lack of efficacy of peptides inserted in the fiber knob could be due to this difference when the peptides are identified. Furthermore, the lack of efficacy would be dependent on conformational changes after ligation of the peptide to the fiber knob. To overcome these limitations, Pereboev *et al.* employed a modified filamentous phage-displayed system, pJuFo, which was originally designed to display C-terminal protein fragments (Pereboev *et al.*, 2001). They developed a system for displaying peptides in the context of the fiber knob on the surface of the phage. A display system based on phage λ, which expresses a functional Ad fiber knob on the surface, was also developed (Fontana *et al.*, 2003). By using these systems, Ad vectors containing novel peptide ligands were generated, transducing NIH3T3 and dendritic cells at 100- to 1000-fold higher efficiency than conventional vectors (Fontana *et al.*, 2003). The development and evaluation of the next generation of targeted vectors by incorporating the novel peptides into native tropism-ablated Ad vectors is expected. In the case of the adeno-associated virus (AAV) vector, a method for incorporating random small peptides in the viral capsid has been developed (Muller *et al.*, 2003). This type of screening for ligands might be useful for targeted Ad vector, although the creation of an Ad library with wide diversity is a challenge.

Propagation of modified Ad vectors that no longer bind with cellular receptors (CAR, $\alpha_v$ integrin, and heparan sulfate) requires a special packaging cell line. Two types of packaging cell lines have been reported. One utilizes 293 cells modified to express an artificial receptor molecule (Douglas *et al.*, 1999; Roelvink *et al.*, 1999) that should not have any natural analogs, such as the anti-His single-chain antibody (scFv) and anti-hemagglutinin (HA) scFv. The other approach is to use 293 cells expressing Ad5 fiber protein (Fiber-293 cells) (Von Seggern *et al.*, 1998; Legrand *et al.*, 1999; Koizumi *et al.*, 2003a). In the case of cell lines expressing anti-His scFv, a His tag sequence has been introduced into the C-terminal region of the fiber knob in Ad vectors (Douglas *et al.*, 1999), whereas in the case of cell lines expressing anti-HA scFv, an HA tag sequence has been introduced into the HI loop of the fiber knob or the penton base instead of the RGD motif (Roelvink *et al.*, 1999). Modified Ad vectors are generated by interaction of the tag sequence in the virus with the scFv against the tag sequence on the cells. When the modified Ad vectors are propagated in Fiber-293 cells, wild-type fibers are incorporated in the virus during amplification, resulting in the virus containing both wild-type fibers and mutated fibers. This virus infects 293 (Fiber-293) cells via the wild-type fiber. At the final stage of viral amplification, mutated Ad vectors are allowed to infect normal 293 cells. The recovered viruses should contain only mutant fiber proteins. When Fiber-293 cells have been used as packaging cell lines, either the HI loop or the C-terminal region of the fiber knob as well as the penton base can be used to display a foreign ligand on the vectors. This makes these cells advantageous over cell lines expressing anti-His scFv or anti-HA scFv. In both methods, modified vectors were generated to particle titers similar to that of conventional Ad vectors (Douglas *et al.*, 1999; Roelvink *et al.*, 1999; Koizumi *et al.*, 2003a).

Another strategy to ablate CAR binding by Ad vectors is to proteolytically remove the knob domain of Ad fibers via the insertion of a single factor Xa cleavage site in the fiber shaft, between the cellular ligand and knob domain (Magnusson *et al.*, 2001; Hong *et al.*, 2003; Gaden *et al.*, 2004). As cellular ligands, the RGD peptide and a 58-residue oligopeptide termed the affibody, which binds specifically to the human IgG1 Fc domain, were introduced and ligand-mediated gene transfer was reported (Magnusson *et al.*, 2001; Hong *et al.*, 2003; Gaden *et al.*, 2004).

Ad vectors in which the fiber protein was replaced with phage T4 fibritin were also developed (Krasnykh *et al.*, 2001;

Belousova *et al.*, 2003; Papanikolopoulou *et al.*, 2004). In these vectors, structural similarity between the Ad fiber and bacteriophage T4 fibritin proteins was used, and the fiber shaft and knob domains were replaced with T4 fibritin and a receptor-binding ligand. The human CD40 ligand was functionally displayed in the chimeric fiber of the Ad vectors (Belousova *et al.*, 2003). This approach seems to overcome structural conflicts between the fiber and the targeting ligand.

As described above, several types of vector systems have been developed, using a genetic strategy. These vectors would provide a platform for future targeted Ad vector development. Future efforts should be directed toward novel ligands for specific tissue targeting.

### *Modification by the use of adaptor molecules*

Retargeting of Ad infection can also be achieved through the use of bispecific or bifunctional adaptor molecules composed of an anti-fiber antibody fragment and a cell-binding component. Douglas *et al.* conjugated folate to the neutralizing Fab fragment of an anti-fiber monoclonal antibody (mAb). This Fab–folate conjugate was complexed with an Ad vector and shown to redirect, at high efficiency, the Ad infection of target cells via the folate receptor (Douglas *et al.*, 1999). The Fab fragment of the anti-fiber mAb has been utilized to conjugate with several other ligands including fibroblast growth factor 2 (FGF-2) (Goldman *et al.*, 1997; Sosnowski *et al.*, 1999), epidermal growth factor receptor (EGFR) (Miller *et al.*, 1998), and an anti-CD40 mAb fragment (Tillman *et al.*, 1999). Reynolds *et al.* succeeded in targeting pulmonary endothelial cells *in vivo* by the intravenous injection of Ad vectors complexed with bispecific antibody against the Ad fiber knob and angiotensin-converting enzyme (Reynolds *et al.*, 2000). In a similar strategy, the anti-Ad fiber knob scFv (Watkins *et al.*, 1997; Haisma *et al.*, 2000; Nettelbeck *et al.*, 2001) or the extracellular domain of CAR (Dmitriev *et al.*, 2000; Itoh *et al.*, 2003) was used as the attachment molecule with the virus. Fusion proteins or complexes of ligands with the anti-Ad fiber knob scFv or CAR were used as adaptor molecules (Fig. 1B).

Combination of the adaptor molecule and genetically modified capsids of the Ad vector has also been reported. The Fc-binding domain of staphylococcal protein A was genetically incorporated into the Ad fiber protein (Henning *et al.*, 2002; Korokhov *et al.*, 2003; Volpers *et al.*, 2003). Two studies incorporated the Fc-binding domain into either the HI loop or C terminus of the fiber knob (Korokhov *et al.*, 2003; Volpers *et al.*, 2003), whereas one study incorporated the Fc-binding domain into a knob-deleted fiber containing seven shaft repeats and an external trimerization motif (Henning *et al.*, 2002). Targeting components such as the antibody and fusion protein of the ligand with the Fc domain of immunoglobulin effectively bind to the modified Ad vectors, resulting in specific gene delivery. Because the target-specific ligands such as antibodies are simply changed in this system, these types of Ad vectors should be useful for systematic screening and detection of the target-specific ligands, as well as for therapeutic applications.

Metabolically biotinylated Ad vectors have been developed as another type of vector with adaptor molecule and genetically modified capsid. Barry and colleagues designed a system based on the fusion of a truncated form of the *Propionibacterium shermanii* 1.3S transcarboxylase domain (PSTCD), which functions as a biotin acceptor peptide (BAP) and is efficiently biotinylated by human holocarboxylase synthetase, to the C terminus of the Ad fiber protein (Parrott *et al.*, 2003) or the C terminus of the Ad pIX protein (Campos *et al.*, 2004). In this system, Ad vectors containing BAP are metabolically biotinylated during vector production by the endogenous biotin ligase in 293 cells, resulting in covalently biotinylated virions. Biotinylated Ad vectors are useful as a platform for avidin-based ligand screening and vector targeting by conjugating biotinylated ligands to the virus, using high-affinity tetrameric avidin. Their group performed ligand screening for dendritic cells, using biotinylated Ad vectors (Parrott *et al.*, 2003).

Theoretically, in all the approaches discussed above, any conjugates with one component directed against the Ad capsid (or modified capsid) and the second component directed against the cell surface protein can be applied to increase transduction of target cells. The advantage is that the natural tropism of the fiber knob is usually ablated, possibly as a result of steric hindrance by adaptor molecules. One limitation is that complexes of Ad vectors and adaptor molecules are nonuniform, and batch-to-batch difference of the vectors might occur.

### *Chemical modification by polymers*

Chemical modification with polyethylene glycol (PEG; PEGylation) is frequently used in pharmaceutic preparations to provide a hydrophilic coat and to increase the blood persistence of therapeutic peptides and proteins (Harris and Chess, 2003). Modification of Ad vectors with PEG, in which the activated PEG reacts preferentially with the $\epsilon$-amino terminal of lysine residues on the capsid, including the hexon, fiber, and penton base, prolongs persistence in the blood and circumvents neutralization of the Ad vectors by antibodies (O'Riordan *et al.*, 1999; Romanczuk *et al.*, 1999; Alemany *et al.*, 2000; Croyle *et al.*, 2000, 2001, 2002; Lanciotti *et al.*, 2003; Eto *et al.*, 2004; Ogawara *et al.*, 2004) (Fig. 1C). Furthermore, PEGylated Ad vectors attenuate the ability of the vector to be taken up by antigen-presenting cells, thereby reducing inflammatory responses. Animals administered PEGylated Ad vectors exhibited reduced levels of both cell-mediated and humoral immune responses, resulting in significant gene expression on readministration of unmodified Ad vectors in the lung (O'Riordan *et al.*, 1999; Croyle *et al.*, 2001). However, the PEGylation of Ad vectors leads to loss of infectivity due to steric hindrance by PEG chains (O'Riordan *et al.*, 1999; Alemany *et al.*, 2000; Croyle *et al.*, 2000, 2001, 2002; Lanciotti *et al.*, 2003; Eto *et al.*, 2004; Ogawara *et al.*, 2004). The extent of loss of infectivity and extension of blood retention half-time are dependent on the degree of PEG modification (Eto *et al.*, 2004). The efficiency of transduction of 34% modified PEGylated Ad vectors was approximately 200-fold lower than that of unmodified Ad (Eto *et al.*, 2004).

To overcome the decreased efficiency of infection of PEGylated Ad vectors, vectors containing functional molecules on the tip of PEG have been developed (Lanciotti *et al.*, 2003; Eto *et al.*, 2004; Ogawara *et al.*, 2004). Lanciotti *et al.* reported targeted Ad vectors, using heterofunctional PEG and FGF-2 (Lanciotti *et al.*, 2003). The transduction of Ad/PEG/FGF2 is dependent on the FGF-2 receptor, and is independent of CAR. In

an intraperitoneal model of ovarian cancer, Ad/PEG/FGF2 mediated increased transgene expression in tumor tissue and reduced localization of the vectors to nontarget tissues compared with unmodified Ad vectors (Lanciotti *et al.*, 2003). Ogawara *et al.* reported PEGylated Ad vectors containing E-selectin-specific antibody at the tip of PEG, which target activated endothelial cells (Ogawara *et al.*, 2004). They showed that the systemic administration of PEGylated Ad vectors with anti-E-selectin antibody selectively targeted inflamed skin and mediated local transgene expression in mice with a delayed-type hypersensitivity (DTH) inflammation. As for modification with peptides, $\alpha_v$ integrin-specific RGD peptide-modified PEGylated Ad vectors have been developed (Eto *et al.*, 2004; Ogawara *et al.*, 2004).

Ad vectors coated with polymers other than PEG have also been developed. Seymour's group used a multivalent hydrophilic polymer based on poly[*N*-(2-hydroxypropyl)methacrylamide] to modify Ad vectors (Fisher *et al.*, 2001; Green *et al.*, 2004). Their vector showed an extended plasma circulation time and decreased toxicity, and evaded neutralizing antibodies.

Approaches by chemical modification with polymers are advantageous, in that many ligands, such as peptides, antibodies, and antigens, may be applied to the tips of the polymers. A great deal of knowledge and techniques about chemical modification have been acquired in the study of pharmaceutic preparations for drug delivery systems. Although improved pharmacokinetic properties of polymer (including PEG)-coated Ad vectors without ligands have been reported, those of polymer-coated Ad vectors with ligands have not been reported in detail. The exact nature of those vectors must be characterized further.

## CONCLUSIONS

In this review, we have focused on the development of targeted Ad vectors based on specific virus entry mechanisms. These approaches are easily combined with transcriptional targeting, using tissue/cell-specific promoters. Ideally, combining a better targeted vector containing a modified capsid with a fully deleted Ad genome (i.e., helper-dependent Ad vectors) is desirable to reduce the innate and acquired immunogenicity of the vectors. These combined vectors should be carefully evaluated in terms of transgene expression profile, distribution of the vectors, and pharmacokinetics, including circulation half-life, interaction with blood components, and so on. Anatomical barriers, such as the tightness of endothelial cells, should also be taken into account, because the vectors must pass endothelial barriers to reach target tissues. Although progress still needs to be made in perfecting targeted Ad vectors, steady improvements have been achieved through comprehensive approaches. Targeted Ad vectors are a source of great promise for gene therapy in future, because they enhance gene therapy efficacy and permit the delivery of lower doses, which should result in reduced toxicity.

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RCM

# Improved sensitivity for insulin in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry by premixing α-cyano-4-hydroxycinnamic acid matrix with transferrin

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**This report describes an enhancement of the signal intensities of proteins and peptides in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS). When α-cyano-4-hydroxycinnamic acid (CHCA) premixed with human transferrin (Tf) was used as a matrix, the signal intensity of insulin was amplified to more than ten times that of the respective control in CHCA without Tf. The detection limit of insulin was 0.39 fmol on-probe in the presence of Tf, while it was 6.3 fmol in the absence of Tf. The signal intensity of insulin was also enhanced when the CHCA matrix was premixed with proteins other than Tf (80 kDa), such as horse ferritin (20 kDa), bovine serum albumin (BSA, 66 kDa), or human immunoglobulin G (150 kDa). The optimum spectrum of insulin was obtained when the added amount of protein was in the range 0.26–0.62 pmol, regardless of the molecular weight of the added protein. Tf and BSA outperformed the other tested proteins, as determined by improvements in the resulting spectra. When the mass spectra of several peptides and proteins were recorded in the presence of Tf or BSA, the signal intensities of large peptides such as glucagon were enhanced, though those of smaller peptides were not enhanced. In addition, the signal enhancement achieved with Tf and BSA was more pronounced for the proteins, including cytochrome C, than for the large peptides. This enhancement effect could be applied to improve the sensitivity of MALDI-TOFMS to large peptides and proteins. Copyright © 2004 John Wiley & Sons, Ltd.**

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) and electrospray ionization mass spectrometry have been widely used in studies of protein chemistry, including proteomics studies aimed at sequence identification or quantitative analyses following enzymatic digestion by isotope-coded affinity tags and other tagging systems.[1–8] In particular, MALDI-TOFMS has been used for the qualitative and quantitative analysis of intact proteins.[9–11] When the MALDI technique was first introduced as an ionization method for proteins, a mixture of fine metal powder and glycerol, or nicotinic acid, was used as the matrix.[12,13] Progress has been made with other matrix materials such as sinapinic acid, 2,5-dihydroxybenzoic acid (DHB), and α-cyano 4-hydroxycinnamic acid (CHCA), which have some desirable properties such as less intense adduct peaks and a relative insensitivity to contamination.[14–16] With the MALDI approach, analyte proteins are dispersed on a surface in a thin layer of matrix. The energy of an incident pulse of laser photons is absorbed by the matrix to form a jet of matrix vapor that lifts the analyte proteins from the surface and transforms some of them into ions.[13]

However, the mechanisms by which laser light irradiation is able to generate macromolecular ions have not been fully verified to date. It has been reported that the ionization of macromolecules by the MALDI process is affected by several factors. For example, peptide signal intensity was increased by the use of acetone as the solvent for CHCA matrix instead of employing the commonly used solvent, a mixture of acetonitrile and aqueous 0.1% trifluoroacetic acid (TFA).[17] The signal-to-noise (S/N) ratios for macromolecules are low in DHB matrix, but the addition of suitable additives (fructose, glucose, fucose, or 2-hydroxy-5-methoxybenzoic acid) to the DHB matrix improved its performance in the high molecular mass range.[18–21] In the CHCA and sinapinic acid matrices, the detection of higher molecular weight proteins was improved by using polytetrafluoroethylene (Teflon) as sample support.[22,23]

Recently, we investigated a method of identifying and quantifying proteins in blood using mass spectrometry. During the present study, we discovered that the signal intensity of human insulin was augmented more than 10-fold when transferrin (Tf) was mixed with the CHCA matrix

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solution used for MALDI-TOFMS. This phenomenon was not specific to either insulin or Tf, which suggested that such enhancements could be used more generally to improve the sensitivity of protein analysis with MALDI-TOFMS.

## EXPERIMENTAL

### Materials

Human atrial natriuretic peptide (hANP), glucagon, insulin, insulin-like growth factor-1 (IGF-1), transferrin (Tf), bovine serum albumin (BSA), horse spleen ferritin (106 mg/mL in 0.15 M NaCl), and ProteoMass Peptide & Protein, were purchased from Sigma (St. Louis, MO, USA). Human immunoglobulin G (IgG, 11.3 mg/mL in 0.01 M sodium phosphate, 0.5 M NaCl, pH 7.6) was obtained from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Human insulin, IGF-1, glucagon, and hANP stock solutions were prepared at concentrations of 100 pmol/μL by dissolving them in 0.1% TFA. Tf and BSA stock solutions were prepared at concentrations of 10 mg/mL by dissolving the materials in Millipore deionized water. ProteoMass Peptide & Protein stock solutions, which include bradykinin fragment 1-7, human angiotensin II, synthetic peptide $P_{14}R$, human ACTH fragment 18-39, bovine insulin oxidized B chain, bovine insulin, equine cytochrome C, equine apomyoglobin, rabbit aldolase, and BSA, were prepared at concentrations of 100 pmol/μL each, according to the manufacturer's instructions.

### Sample application and data acquisition

The Tf-mixed CHCA was a 5:1 mixture of the CHCA solution (10 mg/mL in 50% acetonitrile in 0.1% aqueous TFA) and Tf solution (0.10 μg/μL; the final concentration was approximately 8.3 ng/μL), corresponding to 0.21 pmol Tf on each well of the target plate, if not otherwise noted. The control CHCA was a mixture of the CHCA solution and deionized water (5:1). A portion of each sample solution was immediately mixed with an equal volume of the matrix solution with or without Tf, and an aliquot of 2 μL (corresponding to 1 μL of sample solution) was applied to a stainless steel target plate. Mass spectrometric analyses were performed using an AB4700 proteomics analyzer (Applied Biosystems, Foster, CA, USA). The operating conditions were as follows: Nd:YAG laser (355 nm), linear mode, and detection of positive ions. The spectra were generated by signal averaging 50 laser shots into a single spectrum. The signal intensity was obtained after performing background correction and noise reduction using the Data Processor software (Applied Biosystems). This software was also used to determine the detection limit.

To confirm whether or not the matrix solution was at an optimum composition, serially diluted CHCA, DHB, or sinapinic acid solutions (from 10 to 0.078 mg/mL in 50% acetonitrile, 50% 0.1% TFA) were added to the insulin solution (100 fmol/μL). The most intense signal was obtained when 10 mg/mL CHCA was added to the insulin solution.

## RESULTS AND DISCUSSION

Human insulin solution (6.3 fmol/μL) was mixed with an equal volume of Tf-mixed CHCA or control CHCA. When

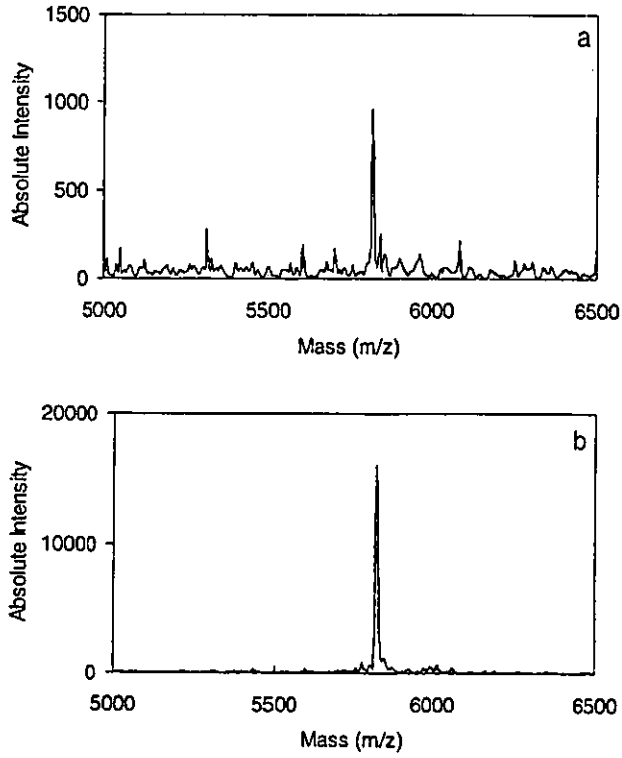


**Figure 1.** MALDI mass spectra of human insulin. The insulin solution (6.3 fmol/μL) and matrix solution were mixed together in equal volumes; 2 μL of the resulting mixture were applied to a target plate, allowed to dry, and analyzed by MALDI-TOFMS (see Experimental). The matrix solution was a 5:1 mixture of CHCA solution (10 mg/mL in 50% acetonitrile in 0.1% aqueous TFA) with deionized water or Tf solution (0.10 μg/μL). (a) Control CHCA used as matrix. (b) Tf-mixed CHCA used as matrix.

the Tf-mixed CHCA was used as matrix, the signal intensity of insulin in the MALDI-TOFMS detection system was amplified more than 10-fold relative to that achieved with the control CHCA (Fig. 1). To assess the sensitivity of insulin detection, the matrix solution was added to serially diluted insulin solutions (from 100 to 0.20 fmol/μL in deionized water), and samples were then spotted on a target plate. The detection limit of insulin was 0.39 fmol on the target plate in a Tf-mixed CHCA matrix under the present experimental conditions, whereas this limit was 6.3 fmol in the case of CHCA without Tf (Fig. 2).

To obtain the optimum concentration of Tf for the enhancement of insulin measurement sensitivity, the CHCA solution was mixed with serially diluted Tf solutions (from 1.0 μg/μL to 7.8 ng/μL) before addition to the insulin solution (100 fmol/μL). The signal intensity increased in a Tf-concentration-dependent manner (Fig. 3(a)). However, the S/N ratio decreased when the Tf concentration was more than 125 ng/μL (Fig. 3(b)), though it should be noted that the S/N value was still higher than the corresponding control value, i.e., 15 ± 7. A signal for 0.39 fmol/μL insulin was detected in the CHCA solution mixed with 0.1 μg/μL Tf (Fig. 2), whereas the signal for 1.6 fmol/μL insulin was not detected in the CHCA solution mixed with 1.0 μg/μL Tf (data not shown). These results suggest that the detection limit was also decreased in the presence of a high concentration of Tf.

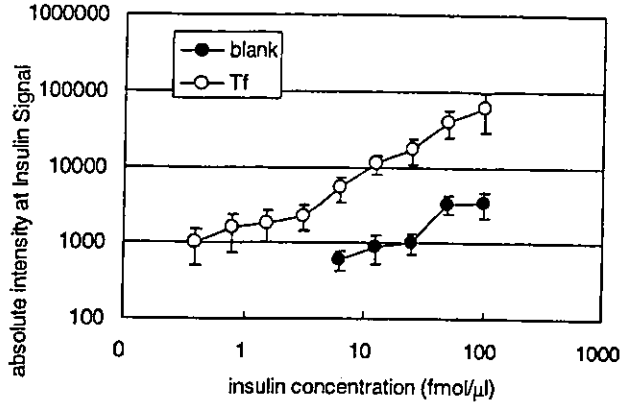


**Figure 2.** Dependence of insulin signals on insulin concentration. Sequentially diluted human insulin solution (100 to 0.20 fmol/μL in deionized water) and matrix solution were mixed in equal volumes. The matrix solution was a 5:1 mixture of the CHCA solution with either deionized water or Tf solution (0.10 μg/μL). The absolute intensity of the insulin signal obtained from Tf-mixed CHCA (open circles) is compared with that obtained for the control CHCA (closed circles). Each point represents the mean ± S.E. of four tests.

It is known that an excess amount of protein components can strongly influence the behavior of the MALDI process, resulting in partial or complete ion signal suppression.[24] In addition, the optimum mass ratio between the analyte and matrix for MALDI analysis has been demonstrated empirically.[15] When the CHCA was mixed with 1.0 μg/μL Tf, the excess amount of Tf might have suppressed the signal intensity of insulin as well. However, if that amount is appropriate, Tf appears somehow capable of enhancing the signal.

To determine whether or not the enhancement of the insulin MALDI-TOFMS signal intensity was specific to Tf, the CHCA solution was mixed with serially diluted solutions of several peptides and proteins before its addition to the insulin solution. The insulin signal intensity was also enhanced in the presence of ferritin (20 kDa), BSA (66 kDa), or IgG (150 kDa) (Fig. 4(a)). However, this was not found to occur in a simple concentration-dependent manner in the case of either ferritin or IgG; furthermore, when the CHCA solution was mixed with more than 2.0 μg/μL of these protein solutions, no insulin signal was detected. The enhancement of the insulin signal intensity was relatively small in the presence of peptides such as hANP (3.1 kDa) and glucagon (3.4 kDa). In addition, when the CHCA solution was mixed with more than 77 ng/μL of hANP or 87 ng/μL of glucagon, no insulin signal was detected. Among the tested peptides and proteins, the insulin signal intensity was enhanced most effectively in the presence of Tf (80 kDa) or BSA. Therefore, it is probable that this type of enhancement requires an added protein of moderate molecular weight, namely 66–80 kDa.

With regard to the results for the serial dilutions of the added peptides and proteins, the highest S/N values were obtained at 4.8 ng/μL hANP, 5.4 ng/μL glucagon, 66 ng/μL ferritin, 0.13 μg/μL BSA, 0.13 μg/μL Tf, or 0.57 μg/μL

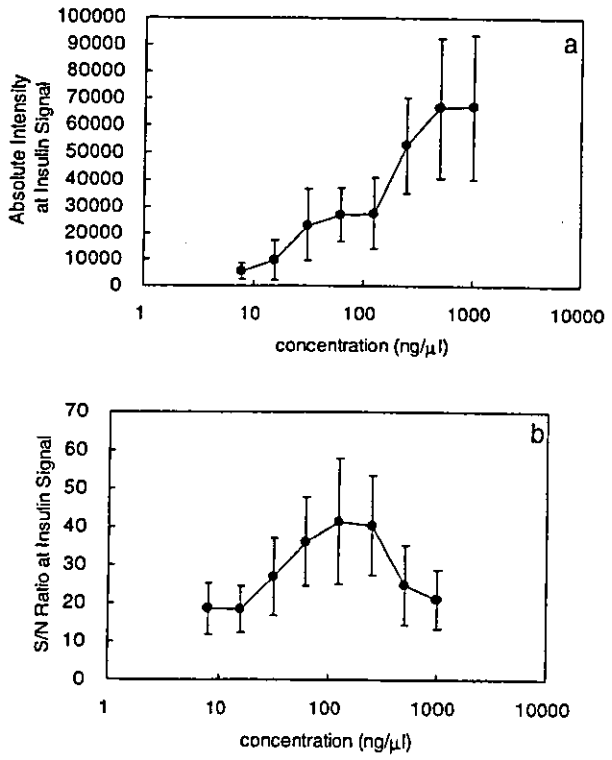


**Figure 3.** Dependence of insulin signal on Tf concentration. Serially diluted Tf solution was added to five volumes of the CHCA solution before mixing the resulting solution with an equal volume of human insulin (100 fmol/μL): (a) absolute intensity (arbitrary units) and (b) S/N ratio of the insulin signal in the MALDI analysis. Each point represents the mean ± S.E. of four tests.

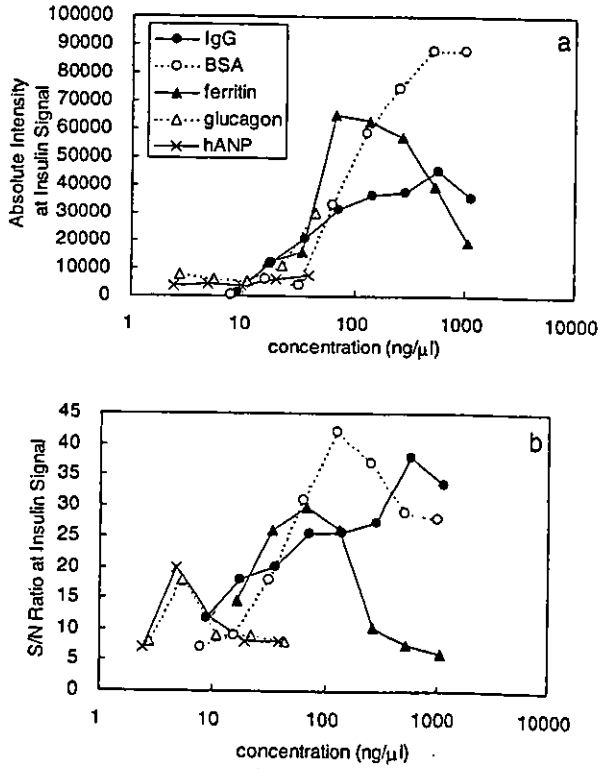


**Figure 4.** Dependence of insulin signal on concentrations of various added proteins. Serially diluted IgG, BSA, ferritin, glucagon, or hANP solution was added to the CHCA solution before the solution was mixed with the human insulin solution (100 fmol/μL): (a) absolute intensity (units) and (b) S/N ratio of the insulin signal. Each point represents the average of duplicate samples.

IgG (Figs. 3(b) and 4(b)), which correspond to 0.26 pmol, 0.26 pmol, 0.50 pmol, 0.32 pmol, 0.26 pmol, and 0.62 pmol, respectively, in each well. Thus, the optimum molar concentrations occurred in the same scale order, although the optimum mass concentrations of polypeptides required to enhance the signal differed markedly between the proteins and small peptides. In addition, the molar concentrations of excess peptides or proteins required to suppress the insulin signal were also found to exhibit the same scale in the same order. The ionization of insulin appeared to depend on the molar concentration of the peptide or protein which was mixed with the CHCA matrix solution.

To examine whether or not the signal enhancement was specific to human insulin, the CHCA solution premixed with Tf or BSA (0.10 μg/μL) was added to a solution of peptides and proteins, which included hANP, glucagon, human insulin, IGF-I, and ProteoMass Peptide & Protein at concentrations of 50 fmol/μL each. The signal intensities of [angiotensin II]$^+$ (1046 Da), [synthetic peptide $P_{14}R$]$^+$ (1534 Da), and [ACTH fragment]$^+$ (2465 Da) were either not enhanced or were reduced in the matrix premixed with Tf or BSA (Table 1). However, the signal intensities of [hANP]$^+$ (3080 Da), [glucagon]$^+$ (3483 Da), [insulin B chain]$^+$ (3494 Da), and [bovine insulin]$^+$ (5730 Da) were enhanced as well as that of [human insulin]$^+$ (5808 Da) (Table 1, Fig. 5). The signal intensities of [IGF-I]$^+$ (7649 Da), [cytochrome C]$^+$ (12 362 Da), and [cytochrome C]$^{2+}$ were enhanced more than that of human insulin in the presence of Tf or BSA. In addition, the signals of [apomyoglobin]$^+$ (16 952 Da) and [apomyoglobin]$^{2+}$ were clearly observed in the presence of Tf or BSA, although their signals were not detected in the control matrix. In this latter case, the signal of [apomyoglobin]$^+$ overlapped with that of BSA, but not of Tf; therefore, it was more advantageous to use Tf than BSA for detecting this signal. Since BSA was included in the ProteoMass Peptide & Protein solution, the signals of [BSA]$^+$ (66 430 Da), [BSA]$^{2+}$, [BSA]$^{3+}$, and [BSA]$^{4+}$ were also detected in the presence of Tf (Table 1, Fig. 5(b)).

The results reported above demonstrate that the enhancement of the signal intensity achieved with the use of Tf and BSA was observed for both peptides and proteins, and this effect was not specific to human insulin. The degree of enhancement was dependent on the molecular weights of the peptides and proteins, and no such enhancement was observed in the case of small peptides; in this regard a dividing line appeared to exist between [ACTH fragment]$^+$ (2465 Da) and [hANP]$^+$ (3080 Da).

The mechanism by which signal intensity enhancement was achieved with the use of peptides and proteins mixed with the matrix solution remains unclear. However, when super DHB (a co-matrix of DHB and 2-hydroxy-5-methoxybenzoic acid) was used as the matrix, ion yields and S/N ratio improved, especially for the high-mass range.[20] It has been suggested that this signal enhancement was caused by a disorder in the DHB lattice, allowing 'softer' desorption. This type of signal enhancement has also been observed in the case of substance P in CHCA after fast evaporation of an acetone solvent, which resulted in the more homogeneous distribution of matrix and analytes.[18] In addition, better mass resolution has been observed in the spectra of cytochrome C in a CHCA matrix desorbed from polyethylene and polypropylene membranes than has been observed with a CHCA matrix desorbed from stainless steel; it was thus suggested that such improved resolution might be due at least in part to the formation of relatively small matrix crystals within the membrane lattice structure.[25] In the present study, Tf and other proteins might have led to a similar disorganization in the CHCA lattice, resulting in the homogeneous distribution of insulin in the CHCA. However, the mechanism may differ from that suggested here, since the disorder in the CHCA lattice cannot reasonably account for why both Tf and BSA were able to enhance the insulin signal more effectively than either hANP or glucagon. As the next step, we are now planning to compare the crystals of the additive macromolecules plus matrix with those of the control matrix, using microscopic examination, to help elucidate the enhancement mechanism. We also intend to investigate whether the enhancement effect is observed in matrices other than CHCA. If crystallization is important,

**Table 1.** Signal intensities for proteins and peptides obtained using a matrix premixed with deionized water or with solutions of Tf or BSA

| | Water | Tf | BSA |
|---|---|---|---|
| [Angiotensin II]$^+$ | 27 834 ± 10 757 | 17 057 ± 5021 | 19 755 ± 11 237 |
| [P14R]$^+$ | 41 689 ± 15 289 | 30 675 ± 8588 | 29 237 ± 13 330 |
| [ACTH 18–39]$^+$ | 4371 ± 1586 | 3801 ± 2246 | 5458 ± 3826 |
| [hNAP]$^+$ | 5158 ± 1323 | 6889 ± 2879 | 9523 ± 6384 |
| [human glucagon]$^+$ | 435 ± 183 | 674 ± 324 | 978 ± 566 |
| [insulin B chain]$^+$ | 367 ± 257 | 997 ± 251 | 715 ± 479 |
| [bovine insulin]$^+$ | 639 ± 100 | 6266 ± 2736 | 7498 ± 5331 |
| [human insulin]$^+$ | 1267 ± 130 | 13 321 ± 5057 | 12 982 ± 6863 |
| [equine cytochrome C]$^{2+}$ | 166 ± 83 | 5668 ± 1975 | 3460 ± 1442 |
| [human IGF-I]$^+$ | 459 ± 81 | 7667 ± 1808 | 6263 ± 2872 |
| [equine apomyoglobin]$^{2+}$ | nd | 2249 ± 994 | 2217 ± 1087 |
| [equine cytochrome C]$^+$ | 114 ± 43 | 7629 ± 1804 | 4006 ± 1981 |
| [BSA]$^{4+}$ | nd | 52 ± 14 | 2459 ± 604 |
| [equine apomyoglobin]$^+$ | nd | 1347 ± 700 | 2090 ± 1316 |
| [BSA]$^{3+}$ | nd | 155 ± 13 | 3721 ± 1426 |
| [BSA]$^{2+}$ | nd | 114 ± 27 | 3624 ± 1681 |
| [BSA]$^+$ | nd | 25 ± 8 | 634 ± 433 |

Each entry is the average of the most intense signals from four samples. nd: no signal was detected.

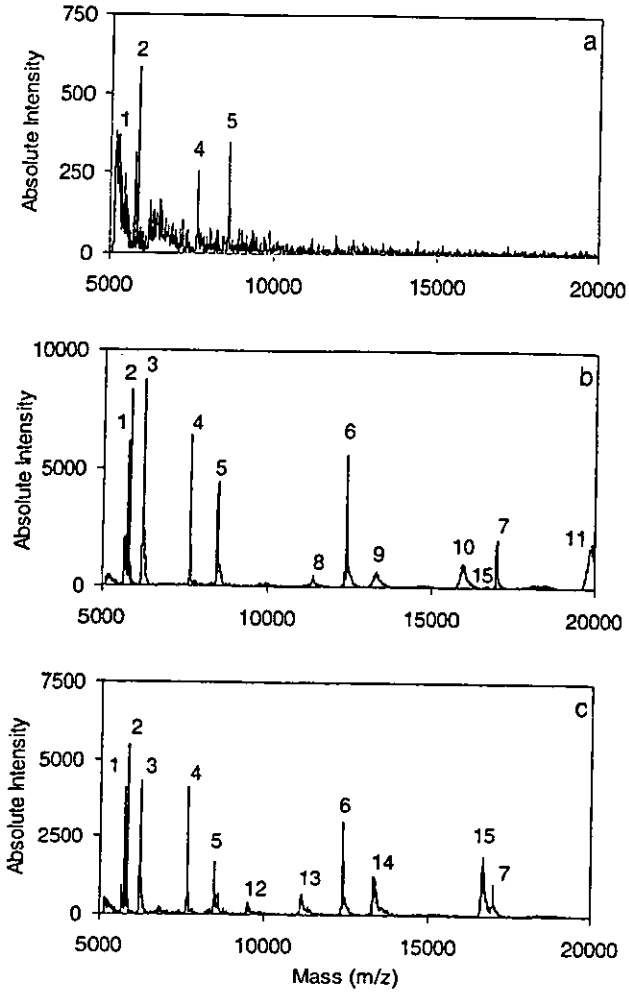


**Figure 5.** MALDI mass spectra of a mixture of peptides and proteins. The mixture of peptides and proteins (50 fmol/μL each) and the matrix solution were mixed together in equal volumes. The matrix solution was a 5:1 mixture of the CHCA solution with (a) deionized water; (b) Tf solution (0.10 μg/μL); and (c) BSA solution (0.10 μg/μL). Signal 1, [bovine insulin]$^{+}$ (5730 Da); 2, [human insulin]$^{+}$ (5808 Da); 3, [cytochrome C]$^{2+}$; 4, [IGF-I]$^{+}$ (7649 Da); 5, [apomyoglobin]$^{2+}$; 6, [cytochrome C]$^{+}$ (12 362 Da); 7, [apomyoglobin]$^{+}$ (16 952 Da); 8, [Tf]$^{7+}$; 9, [Tf]$^{6+}$; 10, [Tf]$^{5+}$; 11, [Tf]$^{4+}$; 12, [BSA]$^{7+}$; 13, [BSA]$^{6+}$; 14, [BSA]$^{5+}$; and 15, [BSA]$^{4+}$.

the effect should not be observed when using liquid matrices.[26,27]

The present results suggest that the enhancement brought about by either Tf or BSA could be applicable to the improvement of sensitivity in the detection of proteins by MALDI-TOFMS in general. However, when Tf or BSA was used as an enhancer in a MALDI-TOFMS system, signals from Tf and BSA were also detected, which sometimes interfered with the analysis of the target proteins. Therefore, neither Tf nor BSA appears to be the best possible enhancer. Further studies are currently underway in order to discover the best macromolecule as an enhancer.

## CONCLUSIONS

We have demonstrated that the signal intensities of insulin and of several peptides and proteins were enhanced in CHCA premixed with Tf or other peptides or proteins. The characteristics of this type of enhancement are as follows: (1) Tf (80 kDa) and BSA (66 kDa) led to better signal enhancement than did small peptides and proteins (<20 kDa) or IgG (150 kDa); (2) the optimum S/N value was observed when the added amount of peptide or protein was within the range 0.26–0.62 pmol; and (3) the signals of peptides of high molecular weight (>3000 Da) were enhanced by the addition of Tf or BSA to CHCA, although the signals of small peptides (<2500 Da) were not enhanced. This type of enhancement may be useful for the improvement of protein analyses with MALDI-TOFMS.

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# Enhancing effect of poly(L-lactide) on the differentiation of mouse osteoblast-like MC3T3-E1 cells

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**Abstract**

Poly(L-lactide) (PLLA) has bioabsorbability and biocompatibility, and it is used as biodegradable screws, pins and plates for internal bone fixation. The purpose of this study was to clarify the effects of low molecular weight (Mw) PLLA on the proliferation and differentiation of mouse osteoblast-like MC3T3-E1 cells. MC3T3-E1 cells were cultured with the concentration of 5–50 μg/ml of PLLA with weight average Mw of 5000 (PLLA-5k) and 10,000 (PLLA-10k) for 2 weeks using the micromass culture. Both PLLAs did not affect the proliferation of MC3T3-E1 cells. However, the calcifications of MC3T3-E1 cells were stimulated with increasing the concentration of the PLLAs. Then PLLA-5k increased the calcification of MC3T3-E1 cells more than PLLA-10k. Additionally, both PLLAs increased the alkaline phosphatase (ALP) activity and calcium content of MC3T3-E1 cells up to the similar level to the calcification. These results indicated that low Mw PLLA enhanced the differentiation of MC3T3-E1 cells with no effect on the proliferation. Moreover, it was suggested that the increase of the ALP activity was a key step to stimulate the calcification of MC3T3-E1 cells. The osteoconductivity of implanted PLLA would be based on the enhancing effect of low Mw PLLA on the differentiation of the osteoblasts.


*Keywords:* Poly(L-lactide); Osteoblast; MC3T3-E1 cell; Calcification; Differentiation; Micromass culture

## 1. Introduction

Poly(L-lactide) (PLLA) with a high molecular weight (Mw) is used as biodegradable screws, pins and plates for internal bone fixation in the orthopedics. Bos et al. reported that the mass loss of PLLA was observed after 26 weeks, and no acute or chronic inflammatory reaction to PLLA was observed until 143 weeks with exception of the early part implant period, by subcutaneous implantation into rats [1]. Otto et al. observed lamellar bone formation around the PLLA wire at 2 and 6 months after intramedullary implantation into rat tibiae [2]. Mainil-Varlet et al. also observed decreasing Mw of PLLA after 4 weeks and bone formation around the PLLA pin at 1 month after implantation into the cortex of sheep tibiae [3]. Thus, there have been many reports on the bioabsorbability and biocompatibility of PLLA.

Recently, there were reported that the lower change in the Mw of PLLA by heat treatment [4] and γ-ray irradiation [5] was responsible for enhancing the differentiation of mouse osteoblast-like MC3T3-E1 cells cultured on the PLLA. It was expected that the low Mw PLLA produced by degradation should enhance the differentiation of osteoblasts. Ikarashi et al. examined the response of MC3T3-E1 cells cultured on several PLLAs with different Mws. The alkaline phosphatase (ALP) activity increased when the cells were cultured on the PLLA with weight average Mw of 20,000 for 2 weeks, but not on the PLLA with Mw of 270,000 and 1,370,000. They also reported that the ALP activity increased when MC3T3-E1 cells were cultured with low Mw poly(DL-lactide) (PDLLA) for 2 weeks [6]. However, the proliferation and differentiation of MC3T3-E1 cells cultured with low Mw PLLA have not been clarified, and it is insufficient to discuss osteoblast differentiation only based on the ALP activity.

In the present study, MC3T3-E1 cells were cultured with low Mw PLLA using the micromass culture, and the differentiation of the cells was synthetically

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evaluated from the ALP activity and calcification. Thus, it was possible to clarify the effects of low Mw PLLA on the proliferation and differentiation of osteoblast-like MC3T3-E1 cells.

## 2. Materials and methods

### 2.1. Materials

PLLA with weight average Mw of 5000 (PLLA-5k) and 10,000 (PLLA-10k) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). The polydispersity indexes, which were calculated as the ratio of the weight average Mw to the number average Mw, of the PLLA-5k and PLLA-10k were, respectively 2.5 and 2.8, by gel permeation chromatography. The PLLAs were used without any refining.

PLLA-5k and PLLA-10k were, respectively, dissolved in dimethyl sulfoxide (DMSO) to a concentration of 50 mg/ml, and sterilized by filtration through a 0.22 μm filter. The sterilized PLLA solutions were serially diluted with DMSO to give concentrations of 5, 10 and 25 mg/ml.

### 2.2. Cells

Mouse osteoblast-like MC3T3-E1 cells were obtained from RIKEN Cell Bank (Saitama, Japan). MC3T3-E1 cells were grown in alpha minimum essential medium (α-MEM) (Gibco Laboratories, Grand Island, New York, USA) supplemented with 20% fetal bovine serum (Intergen, Purchase, New York, USA), 100 μg/ml penicillin and 100 mU/ml streptomycin in a 37°C humidified atmosphere of 5% $CO_2$. The cells were passaged with 0.05% trypsin and 0.1% ethylenediaminetetraacetic acid tetrasodium salts solution (Gibco Laboratories).

### 2.3. Micromass culture

Cell suspensions were prepared in the culture medium and adjusted to give $2 \times 10^6$ cells/ml. A 20 μl aliquot of the cell suspensions was delivered into each well of type I collagen coated 24-well plate (Iwaki Glass, Tokyo, Japan). After the spot-like cells were attached on the well, 1 ml of the culture medium containing 10 mM disodium β-glycerophosphate (β-GP) (Sigma Chemical Co., St. Louis, MO, USA) and 1 μl of the serially diluted PLLA solution was added. As a control, 1 μl of DMSO was added to the culture medium instead of the PLLA solution. The culture medium containing each chemical at the same concentration was changed three times a week, and the cells were cultured for 2 weeks.

### 2.4. Proliferation assay

The proliferation of MC3T3-E1 cells was determined by using a cell proliferation assay reagent, TetraColor ONE (Seikagaku Co., Tokyo, Japan). The cell cultures were exchanged with the culture medium containing 0.1 mM 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, 4 μM 1-methoxy-5-methylphenazinium methylsulfate and 3 mM NaCl, and were incubated for 2 h. The absorbance of the medium was read at 450 nm (reference at 600 nm) with a plate reader (μQUANT, Bio-Tek Instruments, Inc., Winooski, VT, USA). It has been proven that the absorbance and cell population show the linear relationship.

### 2.5. Calcification assay

The calcium depositions of MC3T3-E1 cell cultures were stained by alizarin red S solution. The alizarin red S solution was freshly made; 0.1 ml of 28% ammonia solution in 100 ml distilled water was added to 1 g of alizarin red S in 100 ml distilled water to make it pH 6.36–6.40. After the proliferation was determined, the cell cultures were washed three times with Dulbecco's phosphate-buffered saline without calcium and magnesium salts (PBS(−)) and fixed by the addition of 10% formalin dissolved in PBS(−) solution. After fixing, the cell cultures were washed five times with distilled water and stained by alizarin red S solution for 5 min. The transmission digital images of alizarin red S stained cultures were obtained with a color image scanner (GT-9500WIN, SEIKO EPSON Co., Nagano, Japan) with a transparency unit (GT95FLU, SEIKO EPSON Co.), and then, the alizarin red S stained areas were measured using an image processing and analysis software, Scion Image (Scion Co., Frederick, MD, USA).

### 2.6. Preparation of cell lysates

The ALP activity and calcium content of MC3T3-E1 cells cultured with low Mw PLLA for 2 weeks were measured using the cell lysates [7]. The cell cultures were washed twice with PBS(−). The cells were recovered by trypsinization and washed twice with PBS(−) by centrifugation at 1000 rpm for 2 min. The residues were resuspended in 1 ml of 0.2% Nonidet P-40 and sonicated in an ice bath for 2 min using an ultrasonic processor (VC-50T, Sonic & Materials Inc., Danbury, CT, USA). The cell lysates were stored frozen at −20°C until measurement of the ALP activity and calcium content.

### 2.7. ALP activity

The ALP activity of cell lysates was measured according to the method of Ikarashi et al. [4]. The same

quantity of 2 mM $MgCl_2$ in 0.1 M carbonate buffer (pH 10.2) and 20 mM *p*-nitrophenylphosphate were mixed as the substrate solution, and then, this substrate solution was pre-incubated at 37°C. Twenty microliters of the cell lysates was incubated with 1 ml of the substrate solution at 37°C for 30 min. The enzymatic reaction was stopped by adding 2 ml of 0.25 N NaOH, and the absorbance of *p*-nitrophenol liberated was read at 410 nm. The calibration curve of ALP activity was made by the standard solutions that diluted calf intestine ALP (Boehringer Mannheim GmbH, Germany) at the various concentrations. Total protein content of cell lysates was measured by the method of Lowry et al. [8] with minor modification using bovine serum albumin (Wako Pure Chemical Industries, Ltd.) as a reference standard [5]. The ALP activity of cell lysate was normalized for total protein content of the cell lysate.

### 2.8. Calcium content

The calcium content of cell lysates was determined by using a diagnostic kit, Calcium C (Wako Pure Chemical Industries, Ltd.). The same quantity of the cell lysates and 1 N HCl were mixed, and decalcified for 15 h at room temperature [9]. Ten microliters of the decalcifying solution and 1.0 ml of 0.88 M monoethanolamine buffer (pH 11.0) were mixed, and 100 µl of 0.63 mM *o*-cresolphthalein complexon and 69 mM 8-hydroxyquinoline was added. After 15 min at room temperature, the absorbance of the reaction solution was read at 570 nm. The standard calcium solutions of various concentrations were also operated by the same manner in order to make the calibration curve.

### 2.9. Statistical analysis

All measured values were collected in four sets and expressed in means ± standard deviation (SD). Differences among the groups were evaluated with one-way or two-way analysis of variance (ANOVA). When significant differences among the groups were found, Tukey–Kramer test was applied for multiple comparisons. A *P*-value of less than 0.05 was considered statistically significant.

## 3. Results

### 3.1. Proliferation

Mouse osteoblast-like MC3T3-E1 cells were cultured with the concentration of 5–50 µg/ml of PLLA-5k or PLLA-10k for 2 weeks using the micromass culture. In microscopic observation, the proliferations of MC3T3-E1 cells cultured with PLLA-5k and PLLA-10k were almost the same as that of the control group during the culture period. Fig. 1 shows the effects of PLLA-5k and PLLA-10k on the proliferation of MC3T3-E1 cells. There was no significant difference between the proliferation of the cells cultured with and without PLLA-5k ($P = 0.7537$) and PLLA-10k ($P = 0.7521$) by one-way ANOVA. PLLA-5k and PLLA-10k up to 50 µg/ml did not affect the proliferation of MC3T3-E1 cells.

### 3.2. Calcification

The calcium depositions of MC3T3-E1 cell cultures were stained by alizarin red S solution (Fig. 2). Because alizarin red S combines with the calcium and forms the

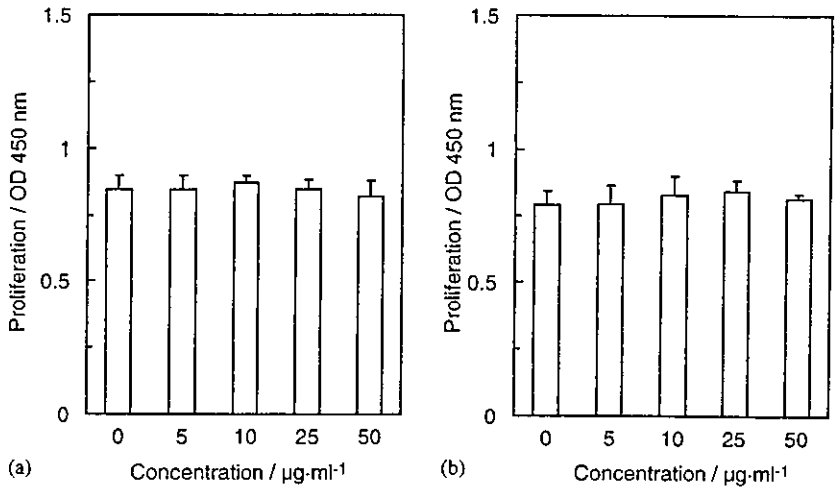


Fig. 1. The effect of PLLA on the proliferation of MC3T3-E1 cells. MC3T3-E1 cells were cultured with the concentration of 5–50 µg/ml of PLLA-5k (a) or PLLA-10k (b) for 2 weeks using the micromass culture. The proliferation of MC3T3-E1 cells cultured with the PLLA was determined using a cell proliferation assay reagent, TetraColor ONE (Seikagaku Co.). Values are means ± SD for four dishes. Significant difference at $P < 0.05$ was not found among the groups by one-way ANOVA.

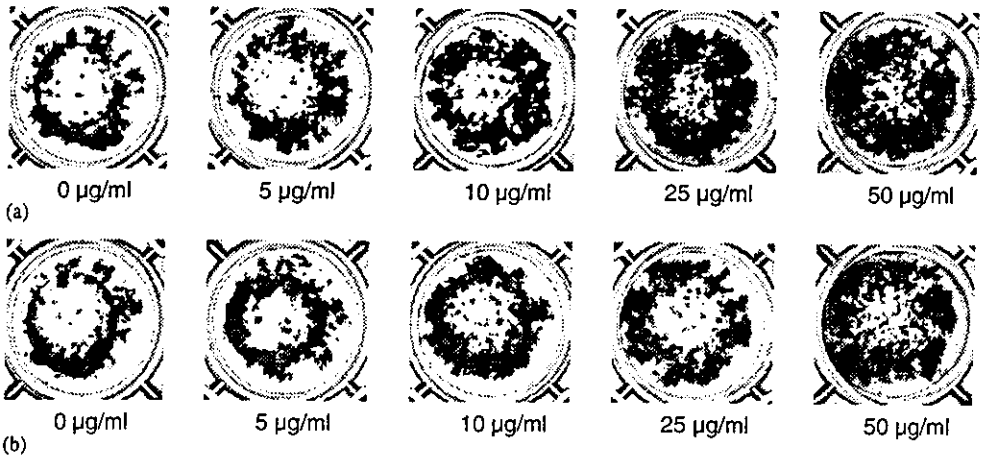


Fig. 2. The appearance of alizarin red S staining of MC3T3-E1 cells cultured with PLLA. MC3T3-E1 cells were cultured with the concentration of 5–50 μg/ml of PLLA-5k (a) or PLLA-10k (b) for 2 weeks using the micromass culture. The culture of MC3T3-E1 cells was stained by alizarin red S solution.

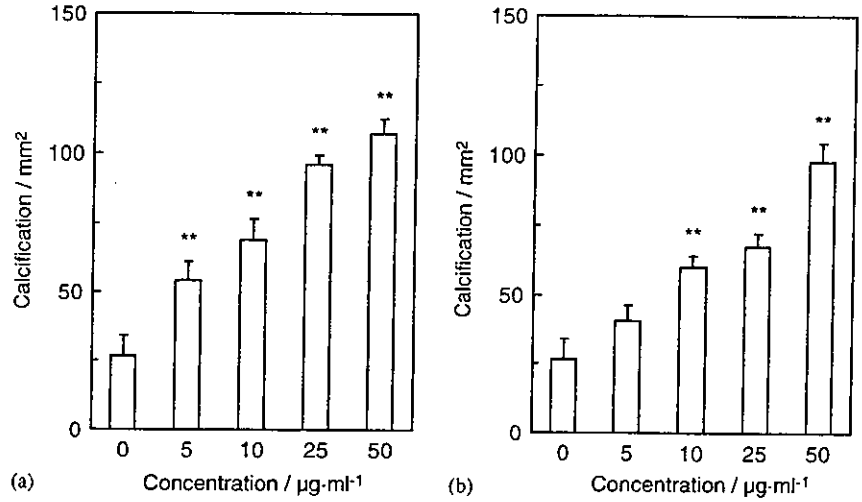


Fig. 3. The effect of PLLA on the calcification of MC3T3-E1 cells. MC3T3-E1 cells were cultured with the concentration of 5–50 μg/ml of PLLA-5k (a) or PLLA-10k (b) for 2 weeks using the micromass culture. The calcification of MC3T3-E1 cells cultured with the PLLA was determined by measuring the alizarin red S stained areas using an image processing and analysis software, Scion Image (Scion Co.). Values are means ± SD for four dishes. Significant difference compared with control (without PLLA) at $^{**}P<0.01$ by Tukey–Kramer test with one-way ANOVA.

lac of poor solubility, the calcification parts in cell cultures are stained dark-red. The calcification parts clearly increased with increasing the concentrations of PLLA-5k and PLLA-10k. The total area of the calcification parts in the well was measured to determine the extent of the calcification of cell cultures. Fig. 3 shows the effect of PLLA-5k and PLLA-10k on the calcification of MC3T3-E1 cells. The calcification of MC3T3-E1 cells cultured with PLLA-5k increased 2.1-fold at 5 μg/ml and increased 4-fold at 50 μg/ml (Fig. 3(a)), and then the calcifications of the cells were significantly increased with increasing the concentrations of the PLLA-5k by one-way ANOVA ($P<0.0001$). On the other hand, the calcification of MC3T3-E1 cells cultured with PLLA-10k increased 1.5- and 3.7-fold at 5 and 50 μg/ml, respectively (Fig. 3(b)), and then the calcifications of the cells were also significantly increased with increasing the concentrations of the PLLA-10k by one-way ANOVA ($P<0.0001$). PLLA-5k and PLLA-10k stimulated the differentiation of MC3T3-E1 cells cultured with the PLLAs, dose-dependently ($A = 11.86\ \mathrm{sqrt}(C_{5k}) + 28.99$, $r = 0.9880$, $P = 0.0016$, $A$: total area of calcification parts ($mm^2$), $C_{5k}$: concentration of PLLA-5k (μg/ml); $A = 9.93\ \mathrm{sqrt}(C_{10k}) + 23.73$, $r = 0.9807$, $P = 0.0032$, $C_{10k}$: concentration of PLLA-10k (μg/ml)). Moreover, when it was compared at the weight concentration of PLLA, PLLA-5k increased the calcification of MC3T3-E1 cells more than PLLA-10k significantly ($P = 0.0075$ by two-way ANOVA).

### 3.3. ALP activity and calcium content

ALP is a representative enzyme of osteoblastic differentiation, and then ALP activity was determined