Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 354 (2008) 23–27
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Mini review

Heat shock protein derivatives for delivery of antigens to antigen presenting cells

Makiya Nishikawa*, Seiji Takemoto, Yoshinobu Takakura

Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

Received 27 July 2007; received in revised form 18 September 2007; accepted 24 September 2007
Available online 29 September 2007

Abstract

Delivery of antigens to antigen presenting cells (APCs) is a key issue for developing effective cancer vaccines. Controlling the tissue distribution of antigens can increase antigen-specific immune responses, including the induction of cytotoxic T lymphocytes (CTL). Heat shock protein 70 (Hsp70) forms complexes with a variety of tumor-related antigens via its polypeptide-binding domain. Because Hsp70 is taken up by APCs through recognition by Hsp receptors, such as CD91 and LOX-1, its application to antigen delivery systems has been examined both in experimental and clinical settings. A tissue distribution study revealed that Hsp70 is mainly taken up by the liver, especially by hepatocytes, after intravenous injection in mice. A significant amount of Hsp70 was also delivered to regional lymph nodes when it was injected subcutaneously, supporting the hypothesis that Hsp70 is a natural targeting system for APCs. Model antigens were complexed with or conjugated to Hsp70, resulting in greater antigen-specific immune responses. Cytoplasmic delivery of Hsp70–antigen further increased the efficacy of the Hsp70-based vaccines. These findings indicate that effective cancer therapy can be achieved by developing Hsp70-based anticancer vaccines when their tissue and intracellular distribution is properly controlled.

Keywords: Heat shock protein; Tumor antigen; Antigen presenting cell; Pharmacokinetics; Tissue distribution; Intracellular trafficking

Contents

1. Introduction

Immunotherapy, in which an antigen-specific immune response is induced, can be a safe and effective therapeutic means of treating cancers and pathogenic infections. Administration of antigens will result in the induction of antigen-specific cytotoxic T lymphocytes (CTLs), which then destroy antigen-expressing target cells, such as cancer cells. In general, the

* Corresponding author. Tel.: +81 75 753 4580; fax: +81 75 753 4614.
E-mail address: makiya@pharm.kyoto-u.ac.jp (M. Nishikawa).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.09.030

induction of a CTL response requires that antigen presenting cells (APCs), such as dendritic cells (DCs) and macrophages, internalize antigens, degrade them and present the resulting peptide fragments to the MHC class I molecule. Because immunotherapy activates the inherent immune system for destroying target cells, it can be considered as a safe therapeutic approach with few side effects compared with chemotherapy or radiotherapy. On the other hand, poor induction of CTL by conventional approaches of immunotherapy is a barrier to achieving significant beneficial effects. The major reasons for poor induction are (i) that antigens are poorly delivered to APCs after administration, (ii) that even if delivered to the cells, antigens are not properly processed for presentation to major histocompatibility complex (MHC) molecules, and (3) that the APCs presenting antigen peptides have little ability to induce CTL. The first two problems can be solved by controlling the tissue and intracellular distribution of antigens after administration.

In a series of investigations using ovalbumin (OVA) as a model antigen protein, we have developed systems to deliver antigens to APCs. Because APCs, such as DCs and macrophages, express several types of scavenger receptors, which recognize macromolecules with a strong negative charge, an OVA derivative modified with succinic anhydride was synthesized (Yamasaki et al., 2002, 2003; Shakushiro et al., 2004). Compared with unmodified OVA, this OVA derivative with a strong negative charge, succinylated OVA, was more efficiently taken up by APCs, induced greater CTL responses, and prolonged the survival time of mice bearing EG7, a lymphoma clone expressing OVA. Another promising approach for delivery of antigens to APCs is their cationization. This is because cationic proteins are efficiently taken up by cells through adsorptive endocytosis based on an electrostatic interaction with negatively charged cell membranes (Apple et al., 1988; Futami et al., 2001; Nishikawa et al., 2002). Although adsorptive endocytosis is a rather non-specific process, cationized antigen proteins are taken up by cells including APCs *in vivo* following local injection. We have demonstrated that a cationized derivative of OVA, prepared by conjugating hexamethylene diamine, is efficiently presented on MHC class I *in vitro* and elicits antigen-specific CTLs after immunization into mice (Ikenaga et al., 2004). In these cases, a large amount of OVA is delivered to APCs through scavenger receptors or adsorptive endocytosis, then the fraction taken up by the cells could be miss-sorted from endosomes to cytoplasm, which would lead to an increased 'cross-presentation' of the antigen to the MHC class I molecule.

A further increase in immune response to cancer cells would be achieved by controlling the intracellular trafficking of antigens in APCs and by activating APCs by some means. Recently, heat shock proteins (Hsps) have attracted great interest as immune activation molecules (Nicchitta, 2003; Reimann and Schirmbeck, 2004; Facciponte et al., 2005). Heat shock protein 70 (Hsp70) is a member of a family of molecular chaperons that are induced under stress conditions (Bukau and Horwich, 1995). Hsp70 and other Hsps are highly conserved peptide-binding molecules and play essential roles in protein folding, degradation of misfolded proteins, and metabolism (Hartl, 1996; Rothman, 1998). In addition to these roles as molecular chaperons, Hsps have the ability to bind antigenic materials within cells through the polypeptide-binding domain (Zhu et al., 1996). In addition, Hsp70 is efficiently taken up by APCs via Hsp receptors, such as CD91 or LOX-1 (Chu and Pizzo, 1993; Binder et al., 2000, 2004; Delneste et al., 2002; Takemoto et al., 2005). Furthermore, Hsp70 can activate the innate immunity through the CD40 and Toll-like receptor (TLR)-2 and TLR-4 with cofactor CD14 and induce cytokine secretion from DCs (Udono and Srivastava, 1994; Basu et al., 2000; Somersan et al., 2001; Srivastava, 2002; Gross et al., 2003). These biopharmaceutical, pharmacokinetic and biological properties of Hsp70 make it a suitable carrier for antigens as far as their delivery for antitumor immunity is concerned. Its application to antigen delivery would induce an effective antitumor immune response through a combination of several functions of the Hsp70–antigen peptide complex (Gullo and Teoh, 2004). Fig. 1 shows the possible roles of Hsp70 and its peptide complex in the immune response. In this article, therefore, we shall review the development of antitumor vaccines using Hsp70 and related proteins, focusing on their tissue and intracellular distribution.

2. Tissue distribution characteristics of Hsp70

2.1. Intravenous injection

Hsps outside cells are known to act as a danger signal. However, the tissue distribution of extracellular Hsps has received little attention. The authors have examined the tissue distribution of mouse Hsp70, a major Hsp, after its intravenous injection into the tail vein of mice (Takemoto et al., 2005).

After intravenous injection at a low dose of 10 μg/mouse, ^{111}In-labeled Hsp70 rapidly disappeared from blood circulation, and accumulated in the liver. Increasing the dose of ^{111}In-Hsp70 to 100 μg hardly changed its tissue distribution, indicating the linearity of the tissue distribution of Hsp70 within the dose range investigated. Recently, the authors have developed a computer program, MOMENT(BS), in which a bootstrap calculation was applied to estimating the histograms of pharmacokinetic parameters, such as the area under the plasma concentration–time curve (AUC) (Takemoto et al., 2006). The pharmacokinetic analysis based on the bootstrap method clearly showed that the tissue distribution of Hsp70 is not a function of the administered dose between 10 and 100 μg/mouse.

Hsp70 also exhibited characteristics of receptor-mediated uptake by cells. Co-administration of an excess amount of unlabeled Hsp70 significantly retarded the blood clearance and hepatic uptake, suggesting the involvement of specific mechanisms in the tissue distribution of Hsp70. LOX-1, one of the scavenger receptors that recognize polyanions, is reported to recognize Hsps (Delneste et al., 2002). LOX-1 is mainly expressed on endothelial cells, fibroblasts, smooth muscle cells, macrophages and DCs (Draude et al., 1999). Pre-administration of polyanions, such as succinylated bovine serum albumin (BSA), maleylated BSA and poly[I], at a high dose of 200 μg significantly inhibited the hepatic uptake of ^{111}In-Hsp70, suggesting that LOX-1 and other scavenger receptors are involved in the hepatic uptake of extracellular Hsp70.

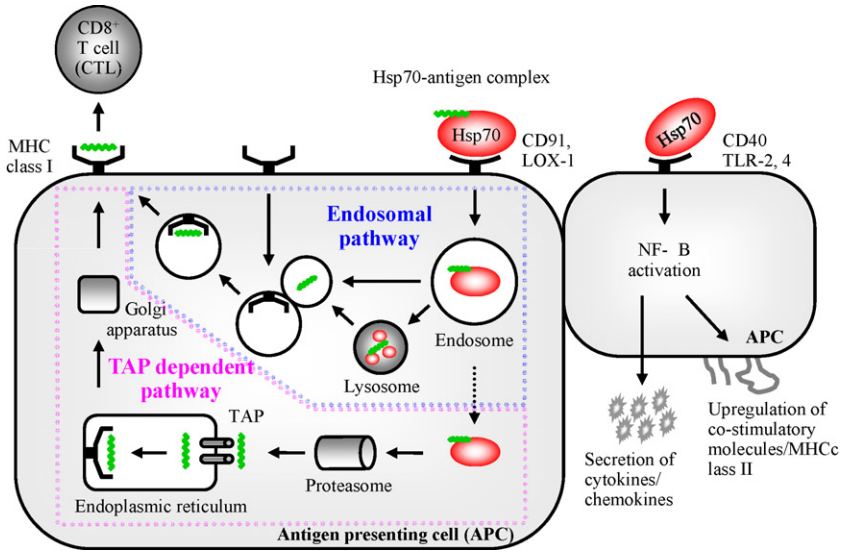

Fig. 1. Roles of Hsp70 in the immune response. Hsp70 is recognized by signaling receptors, such as CD40 and Toll-like receptor (TLR)-2 and TLR-4, on APCs, activates nuclear factor-κB (NF-κB), then upregulates the expression of co-stimulatory molecules and MHC class II and induces the secretion of cytokines/chemokines. In addition, Hsp70 is recognized by Hsp receptors, such as LOX-1 and CD91, and internalized. When antigen peptides are bound or conjugated to Hsp70, the internalized peptides can be processed and presented on MHC molecules. Two pathways, TAP dependent and endosomal pathways, are considered for the presentation of peptides on MHC class I molecules.

CD91, another Hsp receptor, is a member of the lipoprotein receptor family and is expressed on a variety cells, including macrophages, fibroblasts, smooth muscle cells and hepatocytes (Herz and Strickland, 2001). CD91 is also reported to be involved in Hsp-mediated peptide re-presentation by macrophages (Binder et al., 2000). Pre-administration of α_2-macroglobulin, a ligand for CD91 (Basu et al., 2001), significantly inhibited the hepatic uptake of ^{111}In-Hsp70. These results suggest that Hsp70 is delivered to the liver through the recognition of both LOX-1 and CD91.

Separation of liver-constituting cells into parenchymal (hepatocytes) and non-parenchymal cells showed the cell-specificity of the hepatic uptake of Hsp70 (Takemoto et al., 2005). The liver of mice receiving an intravenous injection of ^{111}In-Hsp70 was perfused with a buffer containing collagenase, and the radioactivity in hepatocytes and non-parenchymal cells was measured. The separation revealed that ^{111}In-Hsp70 is predominantly taken up by hepatocytes. The uptake of ^{111}In-Hsp70 in isolated mouse hepatocytes was significantly inhibited by α_2-macroglobulin and anti-CD91 antibody, but not by a control IgG. These results suggest that Hsp70 entering the blood circulation is largely taken up by the liver through recognition by CD91 on hepatocytes.

2.2. Subcutaneous injection

Skin, muscle and tumor tissue are general sites for administration of antitumor vaccines. In general, macromolecules injected into skeletal muscle, skin and subcutaneous tissues are gradually absorbed into blood and lymphatic vessels. ^{111}In-Hsp70 gradually disappeared from the injection site, and a fraction of the ^{111}In-Hsp70 accumulated in the liver with time. In addition, a significant amount of ^{111}In-Hsp70 was also delivered to regional lymph nodes (Takemoto et al., 2005). These tissue distribution characteristics of Hsp70 would make it suitable as a carrier of antigen peptides to APCs.

3. Development of vaccines using heat shock proteins

Hsps have been used in vaccine development for cancer therapy (Blachere and Srivastava, 1995; Gullo and Teoh, 2004; Srivastava, 2005; Aalamian et al., 2006). As described above, Hsps can be recognized by APCs, the target cells for antigen delivery, indicating that Hsps inherently carry a targeting ligand. Therefore, the factors influencing the efficacy of Hsp-based vaccines are: the antigen used, the method of its interaction with Hsps, and intracellular trafficking of Hsp–antigen. Thus far, antigen peptides have been adsorbed onto Hsps through the polypeptide-binding domain, or chemically conjugated to Hsps. In the latter case, such Hsp–antigen conjugates can be designed as a single polypeptide so that its development as a DNA vaccine is also under investigation.

3.1. Induction of immune response by Hsp–antigen

Complex formation of Hsps with antigens has been reported. Endogenous peptides are bound to Hsp70 even after the isolation of Hsp70 from tumor tissues (Binder and Srivastava, 2005). Therefore, once an Hsp70–peptide complex is taken up by APCs, the peptide can be processed and cross-presented by APCs as a tumor-associated antigen. Hsp70–peptide complexes are reported to markedly increase the immune response of APCs to tumor cells compared with free peptide (Javid et al., 2004).

However, the biding affinity of Hsp70 and peptides depends on the peptide sequence (Flynn et al., 1991), which would limit the number and variety of antigens delivered by this approach. Moroi et al. (2000) solved this problem by conjugating a peptide, which has a high affinity for the polypeptide-binding domain of

Hsp70, to antigen. They used a peptide (HWDFAWPW) that has a high affinity for Bip, an Hsp70 homologue present in the endoplasmic reticulum, and conjugated it to an MHC class I antigen of OVA. Immunization with the hybrid peptides complexed to Hsp70 effectively primed specific CTL responses in mice, and led to the rejection of tumors expressing the antigen.

Direct conjugation of Hsps with antigens has also been investigated. Udono et al. (2001) conjugated heat shock cognate protein 70, an Hsp, with an MHC class I antigen of OVA, and succeeded in inducing an antigen-specific CTL response by administration of the conjugate to mice.

3.2. Increase in antitumor effect of Hsp–antigen by controlling its intracellular trafficking

In general, macromolecules are internalized by cells via endocytosis. Hsp70 and its antigen conjugates or complexes can be recognized by Hsp receptors on APCs, then internalized. Once endocytosed, macromolecules are delivered to endosomes then to lysosomes, where they are enzymatically degraded. For immune activation, antigens delivered using Hsps should be presented to the MHC class I molecule, which is a different route from the endocytotic pathway.

We have shown that chemically modified OVAs are more efficiently taken up by APCs than unmodified ones, and this increase in the uptake is effective in inducing an immune response to the antigen (Shakushiro et al., 2004; Ikenaga et al., 2004), probably through acceleration of cross-presentation. These studies indicate the importance of the intracellular trafficking of any challenges of antigen delivery, including the use of Hsp70 as an antigen carrier. Facilitated delivery of antigen from endosomes to cytoplasm would result in an increased anti-tumor immune response through the TAP-dependent pathway (Fig. 1).

Transfer of extracellular macromolecules to the inside of cells has been widely reported in studies aimed at obtaining efficient gene transfer. The endosomal pH is lower than that of cytoplasm, because the proton ATPase on the endosomal membrane pumps protons into the vesicles. If endosomes contain compounds with nitrogen atoms, which can be protonated under an acidic pH, such compounds use up the protons entering the endosomes and the pH drop is inhibited. Then, a further inflow of protons with chloride ions into the endosomes would increase the osmotic pressure, resulting the disruption of endosomes and the release of endosomal contents (Plank et al., 1994; Boussif et al., 1995; Pecheur et al., 1999; Putnam et al., 2001; Zuber et al., 2001). This ‘proton sponge’ effect was reported first with polyethyleneimine, and various compounds, such as polyhistidine, have been reported to have similar effects.

Enhanced cytoplasmic delivery of Hsp70–antigen would increase the entry into the MHC class I presentation pathway, and result in an increased induction of antigen-specific immune response. Laus et al. (2000) reported that the addition of polylysine peptidic sequences and their analogs facilitates antigen penetration into the cytoplasm of APCs, which leads to a significant improvement of MHC class I-dependent antigen presentation to $CD8^+$ T cells. We have selected a polyhistidine as a compound with the proton sponge effect, and designed polyhistidine–Hsp70–antigen conjugates. Intradermal administration of the conjugates to mice resulted in a high CTL response and strong anti-tumor effect compared with Hsp70–antigen conjugates without polyhistidine (Takemoto et al., submitted for publication). These studies indicate the importance of the intracellular trafficking of antigens in order to generate an antigen-specific immune response.

4. Conclusion

Heat shock proteins are endogenous compounds which are recognized by APCs, the target cells for antigen delivery, and can activate these cells. These pieces of evidence indicate that they are highly attractive carriers for antigens to induce an antigen-specific anti-tumor immune response. Clinical studies using Hsp–antigen complexes have already been started, in which Hsp–peptide complexes isolated from tumor tissues of cancer patients are administered to patients (Janetzki et al., 2000; Belli et al., 2002). The therapeutic efficacy of these approaches would be significantly increased by further control of tissue and intracellular distribution of Hsp–antigen complexes or conjugates.

References

Aalamian, M., Fuchs, E., Gupta, R., Levey, D.L., 2006. Autologous renal cell cancer vaccines using heat shock protein–peptide complexes. Urol. Oncol. 24, 425–433.

Apple, R.J., Domen, P.L., Muckerheide, A., Michael, J.G., 1988. Cationization of protein antigens. IV. Increased antigen uptake by antigen-presenting cells. J. Immunol. 140, 3290–3295.

Basu, S., Binder, R.J., Suto, R., Anderson, K.M., Srivastava, P.K., 2000. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kB pathway. Int. Immunol. 12, 1539–1546.

Basu, S., Binder, R.J., Ramalingam, T., Srivastava, P.K., 2001. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. Immunity 14, 303–313.

Belli, F., Testori, A., Rivoltini, L., Maio, M., Andreola, G., Sertoli, M.R., Gallino, G., Piris, A., Cattelan, A., Lazzari, I., Carrabba, M., Scita, G., Santantonio, C., Pilla, L., Tragni, G., Lombardo, C., Arienti, F., Marchiano, A., Queirolo, P., Bertolini, F., Cova, A., Lamaj, E., Ascani, L., Camerini, R., Corsi, M., Cascinelli, N., Lewis, J.J., Srivastava, P., Parmiani, G., 2002. Vaccination of metastatic melanoma patients with autologous tumor-derived heat shock protein gp96–peptide complexes: clinical and immunologic findings. J. Clin. Oncol. 20, 4169–4180.

Binder, R.J., Han, D.K., Srivastava, P.K., 2000. CD91: a receptor for heat shock protein gp96. Nat. Immunol. 1, 151–155.

Binder, R.J., Vatner, R., Srivastava, P.K., 2004. The heat-shock protein receptors: some answers and more questions. Tissue Antigens 64, 442–451.

Binder, R.J., Srivastava, P.K., 2005. Peptides chaperoned by heat-shock proteins are a necessary and sufficient source of antigen in the cross-priming of $CD8^+$ T cells. Nat. Immunol. 6, 593–599.

Blachere, N.E., Srivastava, P.K., 1995. Heat shock protein-based cancer vaccines and related thoughts on immunogenicity of human tumors. Semin. Cancer Biol. 6, 349–355.

Boussif, O., Lezoualc’h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., Behr, J.P., 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. Proc. Natl. Acad. Sci. U.S.A. 92, 7297–7301.

Bukau, B., Horwich, A.L., 1995. The Hsp70 and Hsp60 chaperone machines. Cell 92, 351–366.

Chu, C.T., Pizzo, S.V., 1993. Receptor-mediated antigen delivery into macrophages. Complexing antigen to alpha 2-macroglobulin enhances presentation to T cells. J. Immunol. 150, 48–58.

Delneste, Y., Magistrelli, G., Gauchat, J., Haeuw, J., Aubry, J., Nakamura, K., Kawakami-Honda, N., Goetsch, L., Sawamura, T., Bonnefoy, J., Jeannin, P., 2002. Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. Immunity 17, 353–362.

Draude, G., Hrboticky, N., Lorenz, R.L., 1999. The expression of the lectin-like oxidized low-density lipoprotein receptor (LOX-1) on human vascular smooth muscle cells and monocytes and its down-regulation by lovastatin. Biochem. Pharmacol. 57, 383–386.

Facciponte, J.G., MacDonald, I.J., Wang, X.Y., Kim, H., Manjili, M.H., Subjeck, J.R., 2005. Heat shock proteins and scavenger receptors: role in adaptive immune responses. Immunol. Invest. 34, 325–342.

Flynn, G.C., Pohl, J., Flocco, M.T., Rothman, J.E., 1991. Peptide-binding specificity of the molecular chaperone BiP. Nature 353, 726–730.

Futami, J., Maeda, T., Kitazoe, M., Nukui, E., Tada, H., Seno, M., Kosaka, M., Yamada, H., 2001. Preparation of potent cytotoxic ribonucleases by cationization: enhanced cellular uptake and decreased interaction with ribonuclease inhibitor by chemical modification of carboxyl groups. Biochemistry 40, 7518–7524.

Gross, C., Hansch, D., Gastpar, R., Multhoff, G., 2003. Interaction of heat shock protein 70 peptide with NK cells involves the NK receptor CD94. Biol. Chem. 384, 267–279.

Gullo, C.A., Teoh, G., 2004. Heat shock proteins: to present or not, that is the question. Immunol. Lett. 94, 1–10.

Hartl, F.U., 1996. Molecular chaperones in cellular protein folding. Nature 381, 571–579.

Herz, J., Strickland, D.K., 2001. LRP: a multifunctional scavenger and signaling receptor. J. Clin. Invest. 108, 779–784.

Ikenaga, T., Yamasaki, Y., Shakushiro, K., Nishikawa, M., Takakura, Y., 2004. Induction of cytotoxic T lymphocytes following immunization with cationized soluble antigen. Vaccine 22, 2609–2616.

Janetzki, S., Palla, D., Rosenhauer, V., Lochs, H., Lewis, J.J., Srivastava, P.K., 2000. Immunization of cancer patients with autologous cancer-derived heat shock protein gp96 preparations: a pilot study. Int. J. Cancer 88, 232–238.

Javid, B., MacAry, P.A., Oehlmann, W., Singh, M., Lehner, P.J., 2004. Peptides complexed with the protein HSP70 generate efficient human cytolytic T-lymphocyte responses. Biochem. Soc. Trans. 32, 622–625.

Laus, R., Graddis, T.J., Hakim, I., Vidovic, D., 2000. Enhanced major histocompatibility complex class I-dependent presentation of antigens modified with cationic and fusogenic peptides. Nat. Biotechnol. 18, 1269–1272.

Moroi, Y., Mayhew, M., Trcka, J., Hoe, M.H., Takechi, Y., Hartl, F.U., Rothman, J.E., Houghton, A.N., 2000. Induction of cellular immunity by immunization with novel hybrid peptides complexed to heat shock protein 70. Proc. Natl. Acad. Sci. U.S.A. 97, 3485–3490.

Nicchitta, C.V., 2003. Re-evaluating the role of heat-shock protein–peptide interactions in tumour immunity. Nat. Rev. Immunol. 3, 427–432.

Nishikawa, M., Hasegawa, S., Yamashita, F., Takakura, Y., Hashida, M., 2002. Electrical charge on protein regulates its absorption from the rat small intestine. Am. J. Physiol. 282, G711–G719.

Pecheur, E.I., Sainte-Marie, J., Bienvenue, A., Hoekstra, D., 1999. Peptide and membrane fusion: towards an understanding of the molecular mechanism of protein-induced fusion. J. Membr. Biol. 167, 1–17.

Plank, C., Oberhauser, B., Mechtler, K., Koch, C., Wagner, E., 1994. The influence of endosome-distruptive peptides on gene transfer using synthetic virus-like gene transfer system. J. Biol. Chem. 269, 12918–12924.

Putnam, D., Gentry, C.A., Pack, D.W., Langer, R., 2001. Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. Proc. Natl. Acad. Sci. U.S.A. 98, 1200–1205.

Reimann, J., Schirmbeck, R., 2004. DNA vaccines expressing antigens with a stress protein-capturing domain display enhanced immunogenicity. Immunol. Rev. 199, 54–67.

Rothman, J.E., 1998. Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. Cell 59, 591–601.

Shakushiro, K., Yamasaki, Y., Nishikawa, M., Takakura, Y., 2004. Efficient scavenger receptor-mediated uptake and cross-presentation of negatively charged soluble antigens by dendritic cells. Immunology 112, 211–218.

Somersan, S., Larsson, M., Fonteneau, J.F., Basu, S., Srivastava, P., Bhardwaj, N., 2001. Primary tumor tissue lysates are enriched in heat shock proteins and induce the maturation of human dendritic cells. J. Immunol. 167, 4844–4852.

Srivastava, P.K., 2002. Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. Annu. Rev. Immunol. 20, 395–425.

Srivastava, P.K., 2005. Immunotherapy for human cancer using heat shock protein–peptide complexes. Curr. Oncol. Rep. 7, 104–108.

Takemoto, S., Nishikawa, M., Takakura, Y., 2005. Pharmacokinetics and tissue distribution mechanism of mouse recombinant heat shock protein 70 in mice. Pharm. Res. 22, 419–426.

Takemoto, S., Yamaoka, K., Nishikawa, M., Takakura, Y., 2006. Histogram analysis of pharmacokinetic parameters by bootstrap resampling from one-point sampling data in animal experiments. Drug Metab. Pharmacokinet. 21, 458–464.

Udono, H., Srivastava, P.K., 1994. Comparison of tumor-specific immunogenicities of stress-induced proteins gp96, hsp90, and hsp70. J. Immunol. 152, 5398–5403.

Udono, H., Yamano, T., Kawabata, Y., Ueda, M., Yui, K., 2001. Generation of cytotoxic T lymphocytes by MHC class I ligands fused to heat shock cognate protein 70. Int. Immunol. 13, 1233–1242.

Yamasaki, Y., Sumimoto, K., Nishikawa, M., Yamashita, F., Yamaoka, K., Hashida, M., Takakura, Y., 2002. Pharmacokinetic analysis of *in vivo* disposition of succinylated proteins targeted to liver non-parenchymal cells via scavenger receptors: importance of molecular size and negative charge density for *in vivo* recognition by receptors. J. Pharmacol. Exp. Ther. 301, 467–477.

Yamasaki, Y., Hisazumi, J., Yamaoka, K., Takakura, Y., 2003. Efficient scavenger receptor-mediated hepatic targeting of proteins by introduction of negative charges on the proteins by aconitylation: the influence of charge density and size of the proteins molecules. Eur. J. Pharm. Sci. 18, 305–312.

Zhu, X., Zhao, X., Burkholder, W.F., Gragerov, A., Ogata, C.M., Gottesman, M.E., Hendrickson, W.A., 1996. Structural analysis of substrate binding by the molecular chaperone DnaK. Science 272, 1606–1614.

Zuber, G., Dauty, E., Nothisen, M., Belguise, P., Behr, J.P., 2001. Towards synthetic viruses. Adv. Drug Deliv. Rev. 52, 245–253.